Buffering During Metallic Iron Assisted Autotrophic Denitrification: Role of Pyrite

A Thesis Submitted

In Partial Fulfillment of the Requirements

for the Degree of

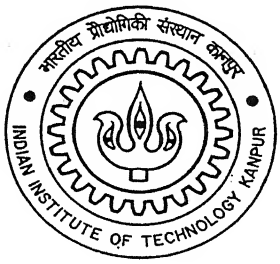
MASTER OF TECHNOLOGY

in

Environmental Engineering and Management

By

Deepti Jha



to the

DEPARTMENT OF CIVIL ENGINEERING

Indian Institute of Technology, Kanpur

July, 2004

Dedicated to....

My
Beloved
Parents, Sister & Friends

CERTIFICATE

It is certified that the work contained in the thesis entitled **"Buffering During Metallic Iron Assisted Autotrophic Denitrification: Role of Pyrite"**, by Miss Deepti Jha, Roll No. Y211706 has been carried out under my supervision and that this work has not been submitted elsewhere for a degree.

Purnendu Bose

Dr. Purnendu Bose
Assistant Professor
Department of Civil Engineering
Indian Institute of Technology
Kanpur

July, 2004

Acknowledgement

First and foremost, I take the opportunity to express my sincere indebtness and profound sense of gratitude to Dr. Purnendu Bose for suggesting me one of the pertinent problems in water pollution and providing me constant guidance and help to come up with a viable solution. His active association, constant encouragement, untiring labor and generous efforts enabled me to give the final shape to my work. His patient attitude and deep insight into the problems in the course of investigation is worth remembering. His ever willingness to help at any moment is beyond comparison.

I am grateful to Dr. Vinod Tare, Dr. Saumyen Guha, Dr. Mukesh Sharma, Dr. Malay Chaudhury, Dr. Deepak Ghosh, Dr. Binayak Rath and Dr. Sachchida Nand Tripathi for providing me vital guidance throughout the course work and otherwise too. They were always accessible and understanding whenever I approached them.

Thanks are due to Mishraji, Dr. R. B. Lalji and Vijayji for their help in their laboratory. It would not have been possible to carry out the experiments without their active support.

The help extended by Harishankar bhaiya, Dilip and others is worth remembering.

I acknowledge the help rendered from our PhD scholars Shuklaji, Satvatji, Alok Sinhaji, K. D. Yadavji, Pawanji , Paritosh, Manoj for their cooperation in the laboratory while working.

I am thankful to Arun, Devendra, Rajveer and Subhankar for providing me help and support during my experiments. The company of Abhinaya, Amita, Bharathi, Bhawana,

Indrani, Leena, Shrawan, Major L. K. G. Singh and Prabhat along with my seniors and juniors will always remind me of the joyful days at IIT Kanpur.

My acknowledgement cannot be complete without mentioning Arvind, Rinku bhaiya and Vinod. I shall never forget the help and support provided by them.

My friendship with Rajneesh, Tripti, Sangita di and Himanshu are the few things at IITK I would cherish for long. Their affection and encouragement throughout my stay in IITK is unforgettable.

Finally, I would like to express my deepest gratitude to my parents and Bulbul, for their constant inspiration. I can hardly pay off their sacrifice.

Thanks to all the people who helped me directly or indirectly throughout the thesis work and helped me in completing this dissertation successfully.

Deepti

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Metallic iron assisted autotrophic denitrification, i.e., denitrification process where microorganisms use hydrogen generated by the anaerobic corrosion of zero-oxidation state iron, Fe(0), as the energy source, inorganic carbon as food source and nitrate as the terminal electron acceptor, is feasible under natural conditions. Practical applications of this process are however limited by the requirement that abiotic reduction of nitrate to ammonia by metallic iron is minimized during biological denitrification, and that suitable buffering is provided to arrest pH increase during the denitrification process. Based on earlier research carried out in our laboratory, it was determined that among various types of metallic iron, commercially available 'steel-wool' showed the least propensity to abiotically reduce nitrate to ammonia, probably due to its low specific surface area. In experiments involving determination of the extent of denitrification during flow through reactive porous media, i.e., sand, seeded with steel wool and denitrifying microorganisms, optimal media composition was determined to be 0.25 g steel wool per 125 cm^3 of sand. When water containing 40 mg/L nitrate (as N) was passed through this media at a retention time of 26 days, the effluent ammonia and nitrate concentrations were below 2 mg/L and 5 mg/L respectively. The objective of this study was to evaluate the suitability of pyrite (FeS_2) as a buffering agent for arresting pH increase during denitrification. Pyrite is considered promising for this purpose because it is a mineral which is unstable under moderately reducing, i.e., anoxic conditions, where it consumes hydroxide ions produced due to denitrification reactions and slowly gets oxidized to ferrous hydroxide $Fe(OH)_2$. The theoretical basis for this buffering action was established through chemical speciation studies using the chemical speciation software, MINEQL+. Experimental evaluation of the buffering efficiency of pyrite showed that it was effective in arresting pH increase associated with denitrification in both batch systems and during flow through reactive porous media. Further, addition of pyrite had no toxic effect on the denitrifying microorganisms, though elevated sulfate concentration was seen in the effluent after denitrification.

CHAPTER I
INTRODUCTION

Various researchers have shown that metallic iron assisted autotrophic denitrification, i.e., denitrification process where microorganisms use hydrogen generated by the anaerobic corrosion of zero-oxidation state iron, Fe(0), as the energy source, inorganic carbon as food source and nitrate as the terminal electron acceptor, is possible. The process may be suitable for a variety of situations, including for 'in-situ' applications as reactive barrier/media for remediation of nitrate contaminated groundwater resources. Practical applications are however limited by the requirement that abiotic reduction of nitrate to ammonia by metallic iron is minimized in such situations, and that suitable buffering is provided to arrest pH increase during the denitrification process.

Based on earlier research conducted in our laboratory, it was determined that among four types of metallic iron tested i.e. 'iron powder', 'iron shaving', 'iron filling' and 'steel-wool', commercially available 'steel-wool' showed the least propensity to abiotically reduce nitrate to ammonia, probably due to its low specific surface area. In semi-batch experiments using up-flow columns containing 0.5 g of 'steel-wool' mixed with 125 cm^3 of sand and seeded with hydrogenotrophic denitrifying microorganisms, it was demonstrated that provision of retention time of 13 days was sufficient to reduce nitrate concentration from 40 mg/L (as N) to below 2 mg/L. However, under these conditions, the extent of denitrification was only 75 percent and nearly 8 mg/L ammonia was detected in the effluent from the column. Based on these results, further experiments of similar type were conducted in columns containing lower 'steel wool' concentrations to reduce ammonia formation. To compensate for the detrimental effects of lower 'steel wool' concentration on rate of denitrification, retention times in such columns were increased from 13 to 26 days. On analysis of results from these experiments, the optimal media composition was determined to be 0.25 g 'steel wool' per 125 cm^3 of sand at a retention time of 26 days, where ammonia and nitrate concentrations in the column effluent were below 2 mg/L and 5 mg/L respectively.

Hydrogenotrophic dentrification as described above may also result in long-term pH increase inside poorly buffered reactive porous media, leading to inhibition of microbial denitrifying activity at pH greater than 9. Under such circumstances, an option may be to fortify the reactive media with additional buffering capacity. Considering the long-term buffering action required, it is obvious that the buffer chosen must be a solid material, which while being retained inside the reactive media will consume hydroxide ions formed during denitrification, and in the process provide the required buffering.

The objective of the study described in this dissertation is to evaluate the suitability of pyrite (FeS_2) as a buffering agent for arresting pH increase during denitrification in the reactive porous media. Pyrite in considered promising material for this purpose because it is a mineral which is unstable under moderately reducing, i.e., anoxic conditions, where it consumes hydroxide ions produced due to denitrification reactions and slowly gets oxidized to ferrous hydroxide $Fe(OH)_2$, thus arresting pH increase in the reactive media. The theoretical basis for this buffering action and evaluation of the buffering efficiency of pyrite during denitrification experiments of various types are examined in detail in this study.

CHAPTER II
LITERATURE REVIEW

2.1 Sources of Nitrate Pollution

The presence of large amounts of nitrate in groundwater is detrimental to the health of humans and animals consuming such water (Goodrich et al., 1981; Bouchard et al. 1992; Vitousek et al., 1997; Howarth et al., 2000). While natural sources of nitrate contamination of groundwater do exist (Edmunds and Gaye, 1999), higher level of nitrate contamination is usually associated with anthropogenic sources (Foster et al., 1992; Kross et al., 1993; Mueller et al., 1995), i.e., application of nitrogenous fertilizers like urea during agriculture and discharge of sewage containing high concentration of organic nitrogen compounds and ammonia into the environment.

Application of nitrogenous fertilizers to soils is an agricultural necessity to obtain high yields (Reddy and Lin, 2000). Nitrogen rich agricultural run-off from fertilized agricultural field is one of the main anthropogenic sources for nitrate pollution to aquatic environment (Holzmacher et al., 1970; Miller et al., 1974). The applied nitrate loading during fertilization may be as high as 75-300 lbs. (as N)/ acre (Spalding et al., 1978; Till et al., 1998). In addition, the presence of large amounts of organically bound nitrogen (1500-6000 kg/ha) in the top 150 mm of most agricultural soils is reported (Howard, 1985). Modern agricultural practices like intensive irrigation result in the conversion of a major part of this organic nitrogen to nitrate (Young and Gray, 1978). Thus, in agricultural regions nitrate contamination can occur in large areas due to combined effect of fertilization and irrigation practices (Saffigana et al., 1977; Spalding et al., 1978). Fresh water resources viz., streams, rivers and lakes are polluted by surface agricultural runoff, while infiltration of nitrate rich water to the subsurface results in the polluted groundwater resources. All this has raised concern over possible contamination of drinking water supplies when nitrate-contaminated water is used as drinking water source (Keeny and Follett, 1991).

Discharge of domestic and industrial sewage into soil or natural aquatic environment is another major anthropogenic source of nitrate pollution (Watson et al., 1967, Franke and McClymonds, 1972). Organic-nitrogen present in such discharges is converted to ammonia-nitrogen by microbial action and may be further converted to nitrate by biological nitrification reactions. As in case of agricultural runoff, surface discharges of wastewater will tend to pollute natural water bodies, while infiltration into soil will cause groundwater pollution. According to the published reports (USGS-OFR, 1990), annual ground water nitrate loading from domestic sewage in Unites States of America (USA) is 4.24 kg/person/year.

2.2 Health Effects

The adverse health effects of high nitrate levels in drinking water are well documented (Walton, 1951; Fan et al., 1987; Gangolli et al., 1994; Ward et al., 1994; Fan and Steinberg, 1996). The most well known health effects are methemoglobinemia, gastric cancer, and non-Hodgkin's lymphoma. Nitrate has the potential to cause methemoglobinemia, both in infants and also in adults deficient in the enzyme glucose-phosphate dehydrogenase (Challis et al., 1980). In human infants and ruminants, reduction of nitrate to nitrite may occur in the gastrointestinal tract, and nitrite may then be absorbed into the bloodstream and react with hemoglobin, blocking normal oxygen transport, thus causing methemoglobinemia or 'blue baby' syndrome (Paul and Clark, 1996; Atlas, 1997). This disease occurs primarily in infants because of the lower acidity of their gastric juices, which provide a better environment for nitrate-reducing bacteria (Comley, 1945). Nitrite may also react with secondary or tertiary amines to form carcinogenic nitrosamines (Doyle et al., 1997) N-nitrosamines are a group of carcinogenic compounds that have been postulated as a cause of stomach cancer (Mirvish, 1992).

2.3 Potable Water Standards for Nitrate

The drinking water standard set by the United States Environmental Protection Agency (USEPA) (Gayle et al., 1989) for nitrate is 10 mg/L (as N). The European Economic Community (EEC) standard is 50 mg/L (as nitrate), or 11.3 mg/L (as N). Indian

recommended guideline (Manual of Water Supply and Treatment, 1999) for concentration of nitrate in drinking water is 45 mg/L (as nitrate). The maximum acceptable concentration of nitrate for potable water according to the World Health Organization (WHO) is 11.3 mg/L as NO_3^--N or 50 mg/L as NO_3^-.

2.4 Environmental Biochemistry of Nitrogenous Species

Conversion of organic nitrogen to mineral nitrogen forms occurs as a result of biological activity and involves several distinct steps. The first step is called ammonification, where the organic nitrogen is converted to ammonia by heterotrophic microorganisms. The subsequent transformation of ammonia to nitrate is called nitrification and occurs primarily through the combined activities of two groups of autotrophic bacteria, *Nitrosomonas*, which converts ammonia to nitrite, and *Nitrobacter*, which converts nitrite to nitrate. Both anaerobic and aerobic microorganisms are involved in ammonification, whereas nitrifying bacteria are strict aerobes.

Microbial reduction of nitrate can be divided two categories, assimilatory and dissimilatory (Payne, 1973). The product of assimilatory nitrate reduction is ammonia that is incorporated into the cell material. Denitrification is a type of dissimilatory nitrate reduction that is responsible for loss of combined nitrogen from ecosystems. In such cases, nitrate serves as the electron acceptor in the energy metabolism and is converted into various gaseous end products, but principally to molecular nitrogen. In such cases, nitrate and nitrite replace molecular oxygen as the terminal electron acceptors. Thus, such bacteria are anoxic, i.e., they do not need oxygen and tend to thrive in oxygen-poor environments.

Although, denitrification is the primary means by which nitrate removal is achieved in natural environment, accounting for 70-75% of total removal (Benefield and Randall, 1980), assimilatory nitrate removal is also of primary importance in certain cases, especially in marine environments (Koike and Hattori, 1978). Denitrification reactions, as described above, take place primarily in anaerobic or anoxic environment. In fact, observations have shown that the biological denitrification rate is a decreasing function of

oxygen concentration (Nakajima et al., 1984). Both heterotrophic and autotrophic microorganisms have been reported to be responsible for denitrification reactions. Heterotrophic denitrifiers are microorganisms that use organic carbon as the food and energy source and nitrate as electron acceptor for their respiration, thus converting nitrate into nitrogen gas. Reactions representing denitrification using methanol (Mahony et al., 2000), ethanol (Richard et al., 1980) and acetic acid (Frick and Richard, 1985) are given as equations 2.1, 2.2 and 2.3 below.

Methanol: $$5CH_3OH+6NO_3^- \rightarrow 3N_2+5CO_2+7H_2O+6OH^- \quad (2.1)$$

Ethanol: $$5C_2H_5OH+12NO_3^- \rightarrow 6N_2+10CO_2+9H_2O+12OH^- \quad (2.2)$$

Acetic Acid: $$5CH_3COOH+8NO_3^- \rightarrow 4N_2+10CO_2+6H_2O+8OH^- \quad (2.3)$$

Autotrophic organisms use inorganic carbon as food source, and electron donors like hydrogen or reduced sulfur-compounds as energy source to reduce nitrate to nitrogen gas. Reactions representing autotrophic denitrification using hydrogen (Kurt et al., 1987), thiosulfate (Claus and Kutzner, 1985a) and sulfide (Barrenstein et al., 1986) as electron donors are given as equations 2.4, 2.5 and 2.6 below.

Hydrogen: $$2NO_3^-+5H_2 \rightarrow N_2+4H_2O+2OH^- \quad (2.4)$$

Thiosulfate: $$5S_2O_3^{2-}+8NO_3^-+H_2O \rightarrow 4N_2+10SO_4^{2-}+2H^+ \quad (2.5)$$

Sulfide: $$5S^{2-}+8NO_3^-+8H^+ \rightarrow 4N_2+5SO_4^{2-}+4H_2O \quad (2.6)$$

2.5 Removal of Nitrate from Water

Various methods have been applied all over the world for the removal of nitrate from water. All these methods involve passing a nitrate-contaminated stream of water through a unit process or process train for removal of nitrate. The processes utilized for nitrate removal can be broadly classified as physico-chemical and biological processes. Biological processes involve denitrification reactions utilizing either heterotrophic or autotrophic denitrifying microorganisms for the removal of nitrate.

In recent times, attention has been directed towards the possibility of in-situ remediation of nitrate-contaminated groundwater resources through biological denitrification reactions. Measures proposed for promoting such reactions, or enhancing the denitrification rates in subsurface environment includes addition of suitable food source and electron acceptor/donor to the nitrate contaminated groundwater. Another approach may be to pass the contaminated groundwater stream through a 'reactive barrier' capable of mitigating nitrate contamination. Such a barrier may be placed across the groundwater flow, such that the nitrate concentration in groundwater is reduced as the water passes through the barrier.

2.5.1 Physico-Chemical Methods

These include ion exchange, membrane separation and chemical reduction. In ion exchange, NO_3^- is exchanged for either chloride (Cl^-) or bicarbonate (HCO_3^-), depending on the resin used. The disadvantage of this technique is the generation of waste brine containing, NO_3^-, Cl^-, HCO_3^-, and SO_4^{2-} (Rogalla et al., 1990). In the membrane separation technique, passing water through a semi permeable material separates nitrate. However, this technique is expensive (eight times greater than ion exchange process) and produces a concentrate (brine), which must be disposed off separately (Kruithof and Koppers, 1989).

Among more innovative physico-chemical methods, (Murphy, 1991) demonstrated chemical reduction of nitrate to ammonia and nitrogen using aluminum powder. The pH range of this reaction was 9-10.5. Ammonia released as above was removed from the system using air stripping or other ammonia removal methods. Other recent studies have investigated the use of an electro-catalytic reduction process to selectively remove nitrate from groundwater associated with small agricultural communities. This technique may prove useful for removing nitrate and nitrite from groundwater associated with non-point source pollution (Peel et al., 2003). Palomares et al., (2003) studied denitrification of natural water on Pd/Cu catalysts supported on hydrotalcite and alumina, and reported rapid reduction of nitrate. Pintaar (2003) has provided an excellent review on the subject

of catalytic reduction of nitrate in presence of hydrogen in particular, and the subject of catalytic processes for purification of drinking water in general.

2.5.2 Biological Processes

Biological denitrification is another alternative to remove nitrate from drinking water sources. Denitrification results in lower operating costs as compared to ion- exchange and reverse osmosis (Kapoor and Viraraghavan, 1997).

2.5.2.1 Heterotrophic Denitrification

Biological denitrification with heterotrophic microorganisms has been widely and successfully applied to water and wastewater treatment. Numerous substrates, including methanol, ethanol, acetic acid, methane, carbon monoxide (Gayle et al., 1989) have been evaluated as electron acceptors for the heterotrophic denitrification process. Denitrification of drinking water supplies has not been tried on full-scale basis in the United States, but there are several full-scale denitrification plants of this type being operated in Europe (Dahab et al., 1998; Gayle, et al., 1989).

In heterotrophic biological denitrification, facultative microorganisms are contacted with the water supply containing nitrates and an added carbon source in an anoxic (oxygen-free) environment. Under these conditions, the bacteria utilize nitrates as a terminal electron acceptor. In the process, nitrates are reduced to nitrogen gas, which is harmless and can be directly discharged to the atmosphere. The extraneous carbon source is necessary since it supplies the energy required by the microorganisms for respiration and synthesis while serving as an electron donor. Most denitrification studies have used methanol (CH_3OH) as the carbon source. Other carbon sources studied include ethanol, acetic acid, glucose, and other more complex organics. While the types of organic compounds may affect the biomass yield, the choice is generally based on economic comparison. The availability of ethyl alcohol from agricultural sources could make this carbon source a strong candidate for denitrification systems. It should be noted that methanol toxicity is such that it is not recommended as electron donor and carbon source for drinking water denitrification.

Another important factor is the presence of dissolved oxygen in the waters and its inhibiting effects. To effect denitrification, the oxygen concentration must be reduced to a level low enough to avoid inhibition or repression of the enzyme nitrate reductase. Unless dissolved oxygen is removed by chemical addition, the amount of electron donor (organic carbon) added must be equal to that needed to remove the oxygen as well as the nitrate.

The first commercial drinking water denitrification facility in France was constructed in 1983 in Eragny (Philipot et al., 1985). This 80 m^3/h facility required ethanol and phosphate addition before passing the water through a biologically active clay filter. Post denitrification treatment included coagulant addition, activated-carbon/sand filtration, and disinfection. The nitrate concentration in the water was reduced from 68 mg/L to 25 mg/L, and nitrite concentration in the effluent was maintained at less than 0.05 mg/L. Denitrification in up-flow fixed-bed reactor was reported from Chateau London, France in 1983 (Frick and Richard, 1985). This 50 m^3/h facility required acetic acid and phosphate addition for denitrification, followed by coagulant addition, activated carbon filtration, and chlorination. Nitrate concentration in water was reduced from 80 mg/L to 30 mg/L. Nitrite accumulated initially after start-up but decreased to less than 0.1 mg/L after process equilibrium was achieved. A large number of fixed-film denitrification processes similar to ones described above were reported to be in operation in France using methanol, ethanol, or acetic acid as substrates (Gayle et al., 1989). A German study (Sontheimer et al., 1987) on denitrification evaluated post-treatment using $FeCl_3$ to reduce effluent nitrite concentrations following fixed-film denitrification using acetic acid. Other German contributions include a fixed-film biological denitrification process using ethanol (Roennefahrt, 1985), which was commercialized under the trade name 'Denipor'. The process consisted of ethanol and phosphate addition, a fixed-film filter packed with floating 'Styropor' spheres, aerobic biological filtration, and chlorination. 95% nitrate removal efficiency was obtained at a filter-loading rate of 1.0 kg NO_3^-/m^3 per day and at re-circulation rate of 200-500%. But, water quality was not improved by additional post-treatment with ozone and activated carbon or coagulants. Frank and Dott (1985) reported on the performance of a pilot-scale bioreactor packed with polystyrene

beads, using methanol or ethanol as an energy source. Reduction of nitrate concentration from 55 mg/L to 3 mg/L was reported during this study. Nilsson and Ohlson (1982) studied denitrification in a series of bench-scale columns packed with immobilized *Pseudomonas denitrificans* cells. The bacteria were encapsulated in a sodium alginate polymer and ethanol was used as a carbon source. Using four columns in series, the nitrate concentration was reduced from 104 mg/L to 0.1 mg/L. Nitrite accumulated in the effluent from the first three columns, but was reduced to 0.3 mg/L in the effluent from the fourth column. Yull-Rhee and Futts (1978) studied denitrification with methane, using two bench-scale sand columns in series. The first column was seeded with *Methylomonas* species and was purged with methane and air. The *Methylomonas* oxidized methane under aerobic conditions but did not denitrify. The second column was seeded with *Pseudomonas stutzeri* and supplied with the effluent of the first column but with no additional carbon source or air. The *Pseudomonas* was shown to denitrify using the metabolites produced from methane as carbon and energy source, resulting in symbiotic relationship between organisms representing two different trophic groups. Krantzenstein (1982) used methane for denitrification in three-stage process consisting of oxygen removal, denitrification in a biological filter, and re-aeration. Fuchs (1985) received the patent for a denitrification process using carbon monoxide saturated flexible porous carriers. When depleted, the carriers were resaturated with CO and recycled back to biological reactor. Rauschmaier and Barotke (1985) evaluated 'Birch-wood' as a substrate for denitrification. Bacteria used the cellulose in 'Birch-wood' readily and denitrification was achieved. However lignin tended to accumulate. Bullermann and Keidel (1986) evaluated whey as a carbon source for denitrification in a fluidized-bed reactor. Nitrate reduction was achieved, but the denitrification rate was slow. A hybrid ion exchange/biological denitrification process was studied by Van der Hoek and Klapwijk (1988). This process utilized ion exchange for the removal of nitrate from ground water and incorporated biological denitrification as part of the resin-regeneration step. During regeneration a concentrated NaCl (10-15 g/L) or $NaHCO_3$ (25-30 g/L) solution was circulated in a closed loop between the ion-exchange column and an up-flow sludge bed (USB) denitrification reactor. Methanol was used as a substrate for the USB. The strong brine solution regenerated the exchange resin and the USB removed the

nitrate (700 mg NO_3^--N/L) that accumulated in the brine. Removal of the nitrate allowed for reuse of the brine through several regeneration cycles. A sand filter was used in the loop to minimize carryover of biological solids and organic matter to the exchange resin. After regeneration, the exchange resin was disinfected. However, sulfate can interfere with nitrate removal since most anion exchange resins are more selective for sulfate than nitrate.

More recent studies on heterotrophic denitrification include the following. Kesseru et al., (2003) investigated the performance of a continuous-flow pilot bioreactor containing immobilized *Pseudomonas butanovora* cells. The reactor containing composite beads resting on indented plates at four different levels, showed almost 100% reduction in the influent nitrate content of 50 mg/L at the first level of the bio-reactor itself. The pH of the effluent (7.58) never exceeded the acceptable maximum (8.5). Mora et al., (2003) studied the effect of organic carbon shock loading on endogenous denitrification in sequential batch reactors (SBRs). The carbon source was molasses. Three lab scale reactors were operated simultaneously and fed with similar wastewater. The different aeration periods of 0, 15 and 30 minutes were used. The influent load was quickly increased threefold in relation to original concentration. Results indicated that SBR reactors could adequately withstand moderate shock loading. Hsieh et al., (2003) studied the nitrogen removal from wastewater using a continuous flow double-biofilm reactor. Simultaneous nitrification and denitrification were achieved in the biofilm reactor with nitrification efficiency of 96.5% and denitrification efficiency of 82%. Jun et al., (2003) studied removal of nitrogenous and carbonaceous substances by a porous carrier-membrane hybrid process, achieved by introducing membranes in a porous carrier reactor. Wealander and Mattiason (2003) reported efficient denitrification at low temperatures using a suspended porous carrier biofilm process. Bodik et al., (2003) studied nitrogen removal in an anaerobic baffled filter reactor with aerobic post treatment, and reported intensive nitrification and partial denitrification in the system while treating municipal wastewater. Plosz et al., (2003) examined the effect of residual oxygen concentration in the reactor on denitrification rates. Schipper et al., (2004) examined the role of hydraulic constraints on the pretreatment of a denitrifying wall for

nitrate removal from shallow groundwater. Aslan and Turkman (2004) studied the simultaneous microbial removal of endosulfan (α+β) and nitrate in a biodenitrification reactor by using wheat straw as carbon source and support particles.

The review of the heterotrophic denitrification process as presented above, indicate that such processes were generally able to reduce nitrate concentration in water to below the specified standards. However, some drawbacks were also noticed, as mentioned below.

- Growth of heterotrophic bacteria is limited by the source of organic carbon. Arrangement for the supply of organic nutrient (methanol, ethanol, acetic acid etc) is always a problem.
- Concerns exist over possible bacterial contamination of treated water, presence of residual organics in treated water and possible increase in chlorine demand of treated water.
- Heterotrophic denitrification generally produces excessive biomass and soluble microbial products that require subsequent treatment.
- Denitrification using methane or carbon monoxide is still required to be studied further and their denitrification rate is not impressive either.
- The post treatment is necessary to remove the residual organic contaminant and biomass before final discharge.
- Operation and maintenance of denitrification plants was found to be expensive.

2.5.2.2 Autotrophic Denitrification

The energy source of autotrophic denitrifying microorganisms is derived from oxidation-reduction reactions with elements such as hydrogen or sulfur as the electron donor. Autotrophic denitrifiers utilize inorganic carbon compounds (such as, CO_2, HCO_3^-) as their carbon source. Although autotrophic denitrifying bacteria function well in anaerobic conditions, in many cases autotrophic mode of denitrification have been shown by facultative organisms i.e. *Paracoccus denitrificans, Thiobacillus denitrificans*, etc.

Hydrogenotrophic denitrifiers, i.e., organisms utilizing hydrogen as energy source, are ubiquitous in nature (Till et al., 1998, Gamble., 1976), which enhances the potential of

denitrifying water using autotrophic means by using such microorganisms. Autotrophic denitrification in drinking water was studied using hydrogen as energy source in a bench-scale fluidized bed reactor (Kurt et al., 1987). The process was modeled using a double-Monod saturation function. A commercial design for autotrophic denitrification called 'Denitropur' was introduced by Sulzer water and wastewater treatment company (Hellekes, 1986), which incorporated indirect hydrogen saturation, phosphate addition, four packed bed reactors in series, post aeration, coagulant addition, filtration and UV disinfection. Carbon dioxide was added as an inorganic carbon source and to buffer against an alkaline pH shift. At the operating temperature of 10.5 °C, the microorganism growth rate varied from 0.1 to 0.3 per day. The sludge production was approximately 0.2 kg/kg nitrogen removed, on a dry weight basis. Residence time of 1 to 2 hours was required to remove 50 mg/L nitrate. The denitrification rate in the system varied with mass and activity of the biomass. In another study, *Alcaligenes eutrophus*, a hydrogenotrophic denitrifier was immobilized in polyacrylamide and alginate copolymer to evaluate denitrification in fluidized-bed and as well as batch reactors, and to elucidate the rate of autotrophic denitrification for obtaining the appropriate operating conditions for drinking water treatment (Chang et al., 1999). The maximum rate of denitrification was found to be (0.6-0.7 kg-N/m^3/day) in flow through system. Denitrification was limited by hydrogen concentration. Phosphate concentration also affected the denitrification rate in presence of nitrite. High initial nitrite concentration was observed in case of batch reactors. Lee and Rittman (2003) reported the application of a novel hollow-fiber membrane biofilm reactor for denitrification of drinking water.

Autotrophic denitrification with reduced sulfur as the energy source has also been studied in some detail. Overath et al., (1986) studied autotrophic denitrification using columns packed with elemental sulfur and activated carbon. The columns were 100 mm in diameter and 3 m long and were operated at a volumetric loading rate of 30 L/h. After 15 days of start-up period, influent nitrate concentrations were reduced from 35 mg/L to 0 mg/L. Denitrification was also studied in columns packed with various ratios of elemental sulfur and limestone marl by Blecon et al., (1983). Efficiency of denitrification increased in such columns as the particle size of the packed material was decreased.

Efficiency of autotrophic denitrification was studied in a bench scale completely mixed batch reactor using calcium alginate beads maintained in suspension (Lewandowski et al., 1987). The beads were composed of elemental sulfur, calcium carbonate, and *thiobacillus denitrificans* encapsulated in the calcium alginate polymer. Mixing in the reactor was provided by compressed nitrogen gas. Nitrate was reduced from 27 mg/L to 6 mg/L in 7 hours. The initial nitrogen removal rate was 1.6 mg N/L-h and increased to 4.8 mg N/L-h after approximately 4 hours. Nitrite tended to accumulate initially but was later reduced to less than 2 mg/L. Various authors have evaluated the role of reduced-sulfur compounds such as sulfide and thiosulfate for the denitrification of water and domestic or industrial wastewater (Claus and Klutzner 1985b, La Motta and Salgado, 1985, Martin, 1982, Batchelor and Lawrence 1978b). Sulfate was by-product of denitrification using all above reduced-sulfur compounds. It was noticed that in some cases, denitrification of industrial wastewaters containing very high nitrate concentration (up to 6,000 mg/L) resulted in high sulfate concentration and led to sulfate inhibition. In water treatment conditions, where nitrate concentrations are lower, sulfate inhibition would not be expected. Stoichiometrically, the denitrification of 152 mg/L of nitrate using elemental sulfur would yield 250 mg/L sulfate. In such cases, the potability of the water may be affected adversely. Batchelor and Lawrence (1978a) developed a mathematical model to describe denitrification kinetics using elemental sulfur as a substrate. Sulfur diffusion through the bio-film, nitrate diffusion through the bulk solution, and nitrate diffusion through the bio-film were considered as possible rate-limiting steps. The influence of each of these steps on the overall reaction rate varies as the concentration of nitrate in the bulk solution varies. A mathematical model was developed (LeCloirec et al., 1985) for denitrification kinetics of nitrate by *Thiobacillus denitrificans* on a sulfur-calcium carbonate filter. The model considers biomass growth, nitrate removal, nitrite evolution, and consumption of sulfur.

Autotrophic and heterotrophic denitrification in identical columns was compared (Vidal et al., 2002) for removal of nitrate from a closed system. Autotrophic denitrification rate was found to be higher (20.6-39.8 moles/day) as compared to heterotrophic denitrification (9.9-11.2 moles/day). For the heterotrophic system, organic carbon was

found to be the chief controller of the denitrification rate and was also responsible for maintaining anaerobic environment within the reactor. Similarly for the autotrophic system, inorganic carbon was found to be the important parameter. Formation of significant amount of ammonia was also reported in both studies.

Ammonia removal is often achieved using nitrification/denitrification systems. In such systems, nitrifying bacteria oxidize ammonia to nitrate under aerobic conditions, and nitrate is subsequently or simultaneously reduced to nitrogen gas, under anoxic conditions. Sliekers et al., (2002) have described this concept of completely autotrophic process, in which aerobic ammonia oxidizers and anaerobic ammonia oxidizers simultaneously oxidize ammonia to nitrogen gas and a small amount of nitrate. This is achieved in one single reactor, at oxygen-limited conditions, without the production of N_2O or NO. This process has been called CANON (Completely Autotrophic Nitrogen-removal over Nitrite). Cervantes et al., (2001) studied denitrification in UASB reactor using various nitrate-loading rates and C:N ratios, but under acetate and NH_4-N limiting conditions. Ammonium was used as an alternative electron donor in this study, as described above.

Soares (2002) has studied denitrification in packed bed column reactors using bicarbonate and elemental sulfur. Oh et al., (2001) investigated the effects of organic compounds (methanol and landfill leachate) on sulfur utilizing denitrification. In this study, a number of elemental sulfur-containing columns were operated under autotrophic, mixotrophic, heterotrophic conditions for approximately 1 year. The performance of the column indicated that the mixotrophic column had a higher nitrate removal capacity than the purely autotrophic column. It was also reported that under mixotrophic conditions, some portion of nitrate was removed heterotrophically and sulfur-utilizing autotrophic bacteria denitrified the remaining nitrate without inhibition by organics. Lee et al., (2001) had studied the effect of external carbon source and empty-bed-contact-time (EBCT) on simultaneous heterotrophic and sulfur-utilizing autotrophic denitrification. Flere and Zhang (1999) studied the feasibility of sulfur/limestone-based autotrophic denitrification (SLAD) in pond systems for in-situ remediation of nitrate-contaminated surface water.

Under anoxic conditions, some encouraging results were obtained. Zhang and Lampe (1998) studied the SLAD process in batch reactors. The feasibility of the process was demonstrated under both aerobic and anoxic environment. Kerri and Flora (1998) evaluated two cathode materials and the impact of copper on bio-electrochemical denitrification. Koenig and Liu (2001) worked on the kinetic model of autotrophic denitrification in sulfur packed-bed reactors. Autotrophic denitrification of synthetic wastewater by *Thiobacillus denitrificans* in up flow sulfur packed-bed reactors was studied in order to establish process kinetics for prediction of effluent concentration. Qu et al., (2002) demonstrated a combined two-step process of heterotrophic and electrochemical autotrophic denitrification to treat nitrate contaminated drinking water. Performances were studied under different C:N ratios. Qu et al., (2001) reported autotrophic denitrification of groundwater using electrochemical reactions (activated carbon fiber electrode) to produce hydrogen. Mansell et al. (2002) studied hydrogenotrophic denitrification in a micro-porous membrane reactor. Application of limestone to supply alkalinity for the control of pH in autotrophic denitrification reactors, along with the optimum ratio of limestone to sulfur has been studied (Liu and Koening, 2002).

As can be seen from the discussion presented above, various researchers have tried different approaches for enhancement and improvement of autotrophic denitrification reactions for nitrate removal. Based on these discussions, it may be concluded that certain advantages exist with autotrophic denitrification, e.g., limited biomass production and a consequently lower potential for bio-fouling. However, some problems associated with the autotrophic denitrification process remain. These include,

- Availability of hydrogen for hydrogenotrophic denitrification. As per stoichiometry, 0.35 mg/L of dissolved hydrogen is required for complete denitrification of 1.0 mg/L NO_3-N. Provision of this hydrogen is costly and difficult.
- The autotrophic denitrification process is most effective when the pH is maintained near neutral. However pH of the system tends to increase in case of hydrogenotrophic denitrification, and tends to decrease in case of sulfur assisted

systems. Hence buffering of the system is very important for ensuring optimal autotrophic denitrification rates.

- Accumulation of nitrite is sometimes observed during autotrophic denitrification, particularly in batch reactors (Vidal et al., 2002).
- Sulfur based system always have the potential danger of sulfate or sulfide toxicity.

2.5.2.3 Denitrification in Natural Subsurface Environment

Number of attempts has been made to identify and quantify biological denitrification in natural subsurface environment. Vogel et al., (1980) reported a process of very slow denitrification in a confined aquifer by investigating the nitrate, oxygen, nitrogen and argon concentrations and $^{15}N/^{14}N$ ratios in artesian groundwater with radiocarbon ages ranging up to 27,000 years. Denitrification in a sandy aquifer was investigated (Trudell et al., 1986) in a study that involved measuring the vertical decline in nitrate concentration below the water table. In another study, denitrification was determined to be occurring through assay of slurried core material obtained from fresh water sand and gravel aquifer by the acetylene blockage technique (Smith and Duff, 1988). In a study conducted to evaluate the relevance of natural dissolved organic matter (DOM) as a substrate for denitrifiers, DOM leached from soils did not contribute significantly to the natural attenuation of nitrate, primarily because the bioavailability of leached DOM is low (Siemens et al., 2003). Delvin et al., (2003) studied the effects of the provision of various electron donors, i.e., acetate, hydrogen gas, elemental sulfur, thiosulphate, aqueous ferrous iron, pyrite and granulated iron on nitrate transformation rates in sediments from a municipal water supply aquifer. Pauwels et al., (1998) reported a small-scale artificial tracer test performed on a schist aquifer in France, which helped in clarifying the mechanisms for in-situ, pyrite-mediated autotrophic denitrification in the aquifer. Another study at the same site, Pauwels et al., (2000) further elucidated the denitrification mechanisms and the influence of mixing and water chemistry on the process. Kamolpornwijit et al. (2003) investigated the preferential flow path development in permeable reactive barriers causing denitrification and its influence on long term performance of PRB. Ability of pyrites to act as electron donors for microbial

reduction of nitrate in anoxic subsurface environment has been shown to be one of the prime reasons for nitrate reduction in some aquifers (Postma and Boesen, 1991).

2.6 Inspiration for the Present Study

As discussed earlier, autotrophic denitrification can be brought about by using hydrogen as an electron donor and proceeds according to the following reaction

$$2.NO_3^- + 5.H_2 \rightarrow N_2 + 4.H_2O + 2.OH^- \qquad (2.7)$$

Hydrogen is of the most thermodynamically favorable electron donors for nitrate based respiration, and its high diffusivity through biofilms is conducive to enhanced nitrate removal. However, the use of hydrogen in engineered denitrification systems is limited by its relatively high cost, low solubility and hazardous (explosive) properties during handling and storage.

Metallic or zero-valent iron, Fe(0) is inherently unstable in both aerobic and anaerobic environments. In the presence of oxygen, Fe(0) is oxidized slowly to Fe (II) and Fe (III) by the process commonly known as rusting as shown by equations 2.8 and 2.9 below.

Aerobic Iron Corrosion:

$$2\,Fe(0) + O_2 + 2\,H_2O \rightarrow 2\,Fe^{2+} + 4\,OH^- \qquad (2.8)$$

$$4\,Fe^{2+} + O_2 + 10\,H_2O \rightarrow 4\,Fe(OH)_3(s) + 8\,H^+ \qquad (2.9)$$

Fe (0) may also be oxidized in anaerobic environments, resulting in the reduction of water to hydrogen gas, as shown by equation 2.10 below.

Anaerobic Iron Corrosion:

$$Fe(0) + 2\,H_2O \rightarrow H_2 + Fe^{2+} + 2\,OH^- \qquad (2.10)$$

Under the circumstances it is conceivable that corrosion of Fe(0) in anaerobic environments may supply hydrogen that is required for autotrophic denitrification of nitrate as per equation 2.4, resulting in a combined reaction as described by equation 2.11.

Biological Nitrate Reduction:

$$5\,Fe(0) + 2\,NO_3^- + 6\,H_2O \rightarrow 5\,Fe^{2+} + N_2 + 12\,OH^- \qquad (2.11)$$

Based on the results of a study (Till et al., 1998), it was concluded that autotrophic denitrification of nitrate by hydrogen gas produced from iron corrosion as energy source is possible using both pure culture (*Paracoccus Denitrificans*) and a mixed culture of autotrophic denitrifying microorganisms. However certain additional questions were raised during this study, adequate resolution of which will ensure that Fe (0) assisted autotrophic denitrification may be practically feasible. These points were as follows,

- There is a possibility of abiotic reduction of nitrate through direct reaction with Fe(0), as shown by equation 2.12 below.

  Abiotic Nitrate Reduction:

  $$4\,Fe(0) + NO_3^- + 7\,H_2O \rightarrow 4\,Fe^{2+} + NH_4^+ + 10\,OH^- \qquad (2.12)$$

  Hence, end-product distribution of the nitrate removal process may be adversely affected, i.e., more ammonia formed and less nitrogen gas generated, if the abiotic nitrate removal process gains precedence. Proper reaction conditions must be determined to prevent this occurrence.
- Both abiotic and microbe assisted denitrification processes, equations 2.11 and 2.12 above, result in the production of OH^- ions, which may elevate the pH of the system. Hence, long-term buffering must be provided such that pH increase is not excessive and detrimental to the nitrate reduction reactions.

Biswas (2002) conducted studies geared towards answering questions regarding the type and surface area of iron metal used in effecting a favorable end-product distribution, i.e., formation of nitrogen gas as opposed to ammonia as the primary by-product of the nitrate reduction by Fe (0) assisted denitrification. Accordingly, two parameters, whose effect on biological denitrification were studied in detail are, the type of metallic iron used, and Fe (0) surface area. Subsequently, Lavania (2003), continuing the work initiated by Biswas (2002), determined optimal iron surface area requirements for acceptable end-product distribution during hydrogenotrophic denitrification. It was also suggested by Lavania (2003) that the mineral pyrite added in solid form may be a suitable material for providing long-term buffering to counter the pH increase due to hydroxide ion production during the denitrification process. Objective of the present study is to determine the suitability of pyrite in this regard.

CHAPTER III
SCOPE AND OBJECTIVES

Several researchers have already demonstrated feasibility of the metallic iron-assisted autotrophic denitrification process, but its practical application is limited by the requirement that metallic iron mediated abiotic reduction of nitrate to ammonia, an undesirable end product, is minimized, and that suitable buffering is provided to arrest expected pH increase during the denitrification process. Recent studies in our laboratory demonstrated that steel wool exhibited the lowest propensity to abiotically reduce nitrate to ammonia in metallic iron assisted autotrophic denitrification systems. Effective denitrification was also demonstrated during flow of nitrate-contaminated water through reactive porous media, such that influent nitrate concentration of 40 mg/L (as N) was reduced to less than 5 mg/L, with ammonia formation being limited to less than 2 mg/L. However considerable pH increase, and cessation of biological denitrification above pH 9 was observed in the reactive media during the denitrification process described above.

Objective of the present study was to evaluate the suitability of pyrite (FeS_2) as a buffering agent for arresting pH increase during denitrification. Pyrite is considered promising for this purpose because it is a mineral, which being solid, will be retained inside reactive media. It is also unstable under moderately reducing, i.e., anoxic conditions, where it consumes hydroxide ions produced due to denitrification reactions and slowly gets oxidized to ferrous hydroxide $Fe(OH)_2$. Specifically, the major objectives of this study were,

- Establishment of the theoretical basis for buffering action by pyrite through chemical speciation studies.
- Demonstration of buffering efficiency of pyrite in batch denitrification systems sustained by externally applied hydrogen
- Demonstration of buffering efficiency of pyrite in metallic iron assisted batch denitrification systems.
- Demonstration of buffering efficiency of pyrite in metallic iron assisted flow through denitrification systems.

CHAPTER 4
ANALYTICAL METHODS AND EXPERIMENTAL PROCEDURES

4.1 Introduction

Analytical methods used in the study described herein have been discussed in detail in this chapter. In addition, information, including make and model number of instruments used for various analyses have also been mentioned. This is followed by a detailed description of the experimental setups, and the procedure for conducting the experiments using the experimental setups mentioned above.

4.2 Analytical Methods

In this section, a description of the chemicals, glassware, chemical stock solutions, instruments and analytical methods used for the experiments described in this dissertation is provided. Mostly, proven analytical methods were used for measuring various parameters. Wherever non-standard methods are used, full description of the method is provided.

4.2.1 Chemicals and Glassware

Reagent grade chemicals were used for preparing mineral medium required for bacterial culture development, and for various experiments described in this study. Analytical grade chemicals were used for preparing the eluent and various standards for ion chromatographic determinations of nitrate and nitrite. Pyrite (FeS_2) used in this research was purchased from Hindustan Minerals and Natural History, Kolkata, India. This pyrite was powdered and the fraction passing through 150μm and retained by 75μm sieve was used in subsequent experiments. Iron and copper content in pyrite sample was determined by atomic absorption spectroscopy after aqua-regia digestion. Results showed that the pyrite sample was 35 percent pure and contained negligible amounts of copper. All glassware used in the experiments was of 'Borosil' or 'Corning' brand and were thoroughly cleaned to prevent interference and contamination. Hydrogen and nitrogen gas of 'Iolar' grade was used for developing and maintaining bacterial culture and for other experiments.

4.2.2 Preparation of Stock Solutions

All stock solutions and standards were prepared with ordinary distilled or triple distilled water. Triple distilled water, or Milli-Q water was also used for most experiments and analyses described in this research.

4.2.3 Measurement of Nitrate, Nitrite and Sulfate

Nitrate, nitrite and sulfate were measured using an Ion Chromatograph (Metrohm 761), which was equipped with a Phenomenex STAR ION A 300 IC anion column, and conductivity detector with ion suppression. All samples for nitrate/nitrite/sulfate determination were filtered through 0.2 μm filter paper before injection into Ion Chromatograph. The minimum detectable concentration of nitrate, nitrite and sulfate by this method is 0.1 mg/L.

4.2.4 Ammonia Measurement

Ammonia was measured colorimetrically by Nesslerization method (Method No. 417 B, APHA, et al., 1985). A spectophotometer (Spectronic, 20 D^+, India) with Borosil glass absorbance cells having 1 cm path length were used for this purpose. The limit of detection of ammonia by this method is 0.02 mg/L.

4.2.5 pH Determination

pH was measured using a combination pH electrode (Toshniwal CL-51, India) connected to a digital pH meter (Toshniwal CL-54, India).

4.3 Experimental Procedures

Various types of experiments were conducted such that the research objectives of this dissertation as outlined in Chapter III could be realized. The need for maintaining sterile blanks for highlighting microbial activity was realized and hence sufficient numbers of blanks were run along with every type of microbiological experiment. Sufficient care was taken to maintain sterile conditions wherever the experimental protocol so demanded.

4.3.1 Preliminary Experiments

Methodology adopted for development and maintenance of hydrogenotrophic denitrifying bacterial culture used in various experiments described in this study is

performed for determining the buffering efficiency of pyrite is described in this section.

4.3.1.1 Development of Bacterial Culture

A stock culture of autotrophic denitrifying microorganisms was maintained in glass reactors as shown in Figure 4.1 throughout the duration of this study. Mixed culture of purely autotrophic denitrifying microorganisms was already available from earlier studies conducted on related topics. 1000 mL of the mineral medium was prepared with sterilized ground water from IIT Kanpur campus, containing inorganic carbon (HCO_3^-), substrate (NO_3^-), buffer ($H_2PO_4^-$) and other trace nutrients as per Table 4.1. 50 mL mixed culture already available in the laboratory was added to the reactor through the funnel at the top of the reactor. Next, this inlet was closed and the gas

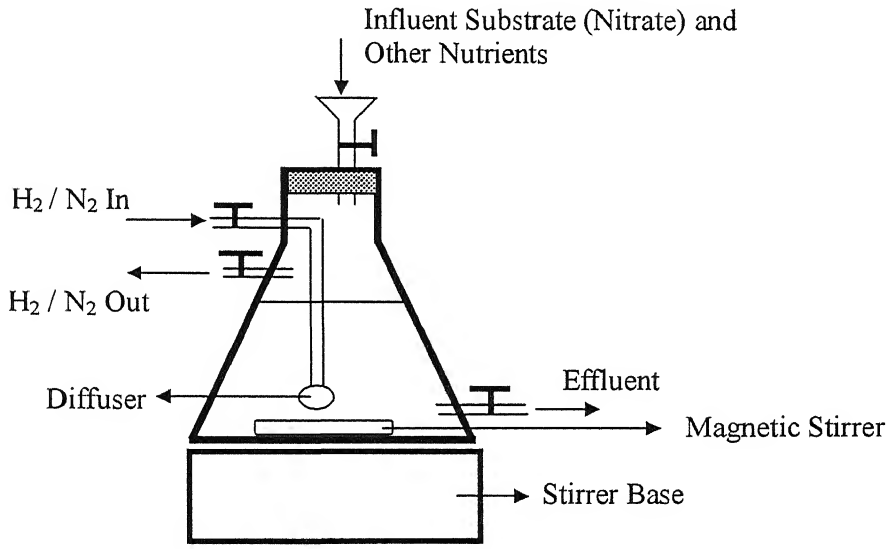


Figure 4.1 Schematic of the Apparatus Used for Developing and Maintaining Mixed Culture of Autotrophic Denitrifying Microorganisms

inlet and outlet to the reactor were opened. Reactor contents were purged with nitrogen gas for 15 minutes to ensure that oxygen was absent in the reactor. Then, hydrogen gas was fed to the reactor by the same procedure for 15 minutes, such that considerable amount of hydrogen gas can be present in the reactor. After this, the gas inlet and outlet ports were closed and the reactor contents were kept in continuously mixed condition by employing a magnetic stirrer. After every six days, reactor

contents were again purged with hydrogen. While purging, 50 mL of reactor contents were withdrawn from the outlet at the bottom of reactor, and 50 mL of the mineral medium containing nitrate introduced from the top of the reactor. This corresponded to a mean cell residence time of 60 days for the microorganisms being cultured in the reactor. The effluent collected as above was analyzed for $NO_3^- - N, NO_2^- - N, NH_3 - N$, pH and absorbance at 600 nm, using 4 cm path length spectroscopic cell (Till et al., 1998). After bacterial growth was established, $NO_3^- - N, NO_2^- - N, NH_3 - N$ concentrations in the effluent, as measured every six days, were nearly zero, while the pH stabilized around 8.5. The absorbance value increased steadily and stabilized, indicating growth of microorganisms and subsequent maintenance of steady state microbial concentration in the reactor.

Table 4.1 Composition of the Mineral Medium (adapted from, Till et al., 1998)

Nutrient	Concentration mg/L
$NaNO_3$	40 (as N)
$NaHCO_3$	250
KH_2PO_4	50
$CuCl_2.2H_2O$	0.0392
$ZnCl_2$	0.1363
$NiCl_2$	0.013
$FeCl_2.4H_2O$	0.7016
$AlCl_3$	0.1106
$MnCl_2.4H_2O$	0.2807
$CoCl_2.6H_2O$	0.0382
$Na_2MoO_4.2H_2O$	0.0254
H_3BO_4	0.0382
Na_2SO_4	0.1420

Next, a second reactor of 3000 mL volume was prepared in a similar way as before. This reactor was seeded with 50 mL of the fully mixed contents of the previous

reactor. This reactor provided seed for all the experiments described in this study. Use of ground water in preparation of the mineral medium ensured that these microorganisms were acclimatized to the groundwater environment, and hence could be used in experiments involving groundwater (Smith et al., 1994).

4.3.1.2 Buffering by Pyrite

Preliminary experiments were carried out to check the buffering capacity of pyrite used in the experiments described herein. For this purpose, 1 g of pyrite was put into a BOD bottle. Then, IITK groundwater, spiked with 40 mg/L nitrate (as N) was sterilized and poured while still warm into the BOD bottle. The bottle was then sealed. The bottled was unsealed every day and one pellet of NaOH, weighing approximately 10 mg was added to the bottle before re-sealing. pH of the solution in the bottle was measured every third day. Another BOD bottle, with no added pyrite, was maintained and analyzed in exactly the same way, but for the purpose of comparison.

4.3.2 Experiment I: Batch Autotrophic Denitrification

Experiment I was carried out in bottles that contained the mineral medium similar to that specified in Table 4.1 but with varying nitrate concentrations. These bottles were seeded with microorganisms from the stock culture reactor, and periodically supplied with hydrogen gas. A typical bottle used for such experiments is shown in Figure 4.2.

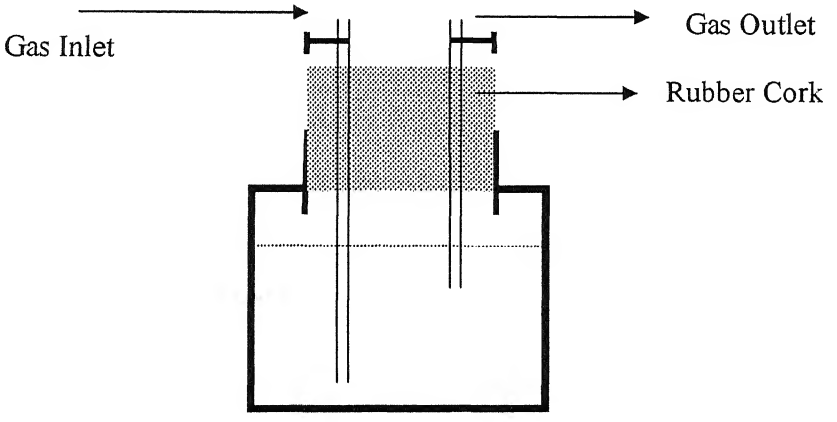


Figure 4.2 Typical Bottle Used for Type I Experiment

A total of 10 bottles were prepared, as specified in Table 4.2. Volume of each bottle was 250 mL. The procedure adopted was as follows. First, 200 mL of the mineral

medium with nitrate concentration as given in Table 4.2 was added to each bottle. Next, 1 g of pyrite was added to five of the bottles, as specified in Table 4.2. Then, all bottles were sealed and sterilized. Then, 5 mL of seed from the stock culture was added to eight bottles, as specified in Table 4.2. Next, the stopcocks on the two tubes of each bottle were opened (see Figure 4.2) and the contents of each bottle were purged with nitrogen gas for 10 minutes so that any residual oxygen in the bottles was removed. Finally, hydrogen gas was passed through each bottle for 5 minutes. Hydrogen gas was applied to the bottles every 3 days, to ensure that the denitrification reaction in these bottles was never hydrogen-limited. Samples were collected from each bottle every 3 days for 45 days. Sample collection involved opening the gas inlet and outlet tubes and applying hydrogen gas pressure through the tube that is not immersed in water. This ensured that liquid sample was ejected from the tube immersed in water. 10 mL of sample was collected from each bottle during each sample collection cycle. The collected samples were sterilized to ensure cessation of all bacterial activity, and stored for measurement of residual $NO_3^- - N$, $NO_2^- - N$ and pH and sulfate.

Table 4.2 Experimental Details for Experiment I

Bottle No.	Nitrate (mg/L as N)	Microorganisms	Pyrite (1 g per bottle)
1	40	No	Yes
2	40	No	No
3	40	Yes	Yes
4	40	Yes	No
5	80	Yes	Yes
6	80	Yes	No
7	120	Yes	Yes
8	120	Yes	No
9	240	Yes	Yes
10	240	Yes	No

4.3.3 Experiment II: Batch Experiments Involving Metallic Iron

Batch experiments were carried out in BOD bottles to determine the rate and extent of metallic iron assisted denitrification. Such experiments were carried out both in the presence and absence of pyrite, in order to determine the efficiency of pyrite in controlling pH increase due to denitrification. Similar experiments were also carried

our under abiotic conditions, i.e., in sterile environment, both in the presence and absence of pyrite, to truly gauge the impact of the presence of hydrogenotrophic denitrifying microorganisms on denitrification. Details of the experiments performed are given in Table 4.3.

Table 4.3 Experimental Details for Experiment II

Expt. No.	Steel Wool (g /300 mL)	Microorganisms	Pyrite (1 g/300 mL)
IIa.	0.1	No	No
		No	Yes
		Yes	No
		Yes	Yes
IIb.	0.25	No	No
		No	Yes
		Yes	No
		Yes	Yes
IIc.	0.40	No	No
		No	Yes
		Yes	No
		Yes	Yes

For abiotic nitrate reduction experiments, BOD bottles were filled with the mineral medium made with sterilized IIT Kanpur groundwater and containing 40mg/L nitrate (as N). 1 g pyrite was added to some bottles, as specified in Table 4.3. Steel wool, i.e., the metallic iron source, was then added to each bottle, as specified in Table 4.3. After this, the bottles were sealed and sterilized again. After the specified time period of reaction, which varied from 10 to 70 days, the bottles were unsealed and samples extracted and sterilized before being stored for analysis of $NO_3^- - N, NO_2^- - N, NH_3 - N$, sulfate, iron and pH.

Typical biological denitrification experiments consisted of filling BOD bottles with mineral medium made with sterilized IIT Kanpur groundwater, but no nitrate. Next, 1 g of pyrite was added to some bottles, as specified in Table 4.3. Then, predetermined quantities of steel wool were added to the bottles, as specified in Table 4.3, after which the bottles were sealed and sterilized. The bottles were left in sealed condition for 7 days to ensure that enough hydrogen has evolved through anoxic corrosion of iron metal to support microbial population. Next, nitrate was added to the bottles

such that nitrate concentration was 40 mg/L (as N) in all bottles. Also, 5 mL of seed from the stock bacteria culture reactor described earlier was added to the bottles. The bottles were then resealed and the contents well mixed. After the specified time period of reaction, which varied from 10 to 75 days, the bottles were unsealed, and the samples extracted and sterilized before being stored for analyzing $NO_3^- - N, NO_2^- - N, NH_3 - N$, pH and sulfate.

4.3.4 Experiment III: Semi-Batch Experiments Involving Metallic Iron

These experiments were conducted in anoxic flow-through columns operated in the up-flow mode. Nitrate contaminated mineral medium was allowed to flow through columns containing sand and various amounts of steel wool, which was the metallic iron source. The columns were kept sterile in case of abiotic experiments and were seeded with microorganisms from the stock culture in case of biological denitrification experiments. The columns were operated in the intermittent-flow mode so that sufficient retention time could be provided in the columns for nitrate reduction reactions to take place. Accordingly, samples were collected once a day for analysis.

The glass columns used for these experiments were approximately 12 cm in length and 4 cm in diameter. These columns were operated in the up-flow mode, with water influent from the bottom of the column. Each column consisted of two parts, a small inlet chamber and a main column with dimensions given above, separated by a porous sintered disk. Water influent to the column first entered the inlet chamber and then passed through the porous sintered disk to reach the main column, which contained the media. The sintered disk also acted as a support for the media in the main column above. The media in the column consisted of sand graded to 1-2 mm diameter. Graded sand was then thoroughly washed with acid and water before drying in an oven. Next, the sand was mixed with varying quantities of steel wool and pyrite, and loaded into the columns. Then the columns were sterilized. The height of media in each column was 10 cm, which corresponded to a media volume of 125 cm^3. Porosity of the media was approximately 50 percent. Each column was sealed at the top by a rubber stopper, with a glass tube passing through it for removing water effluent from the column.

The schematic of the experimental apparatus used in case of abiotic experiments is shown in Figure 4.3. Three abiotic experimental columns were operated simultaneously, with media compositions for these columns being only sand, sand and 0.4 g steel wool, and sand, 1 g pyrite and 0.4 g steel wool, respectively. At the start of a typical abiotic experiment, both chambers of the columns were fully flooded with freshly prepared warm distilled water, which contained little or no dissolved oxygen. The columns were then maintained in sealed condition for 7 days, to ensure onset of anaerobic conditions in the columns. Next, the columns were connected to a reservoir containing mineral medium, prepared as per specifications given in Table 4.1 with IIT Kanpur ground water. In addition to containing approximately 40 mg/L of nitrate (as N), 10 mg/L $HgCl_2$ was also added to the mineral medium in the reservoir to inhibit any microbial activity. Each column was then flushed with approximately 250 mL of mineral medium from the reservoir, so that the nitrate concentration in the effluent from the column was approximately same as the initial nitrate concentration in the reservoir. Then, the valves connecting the reservoir to the columns and those

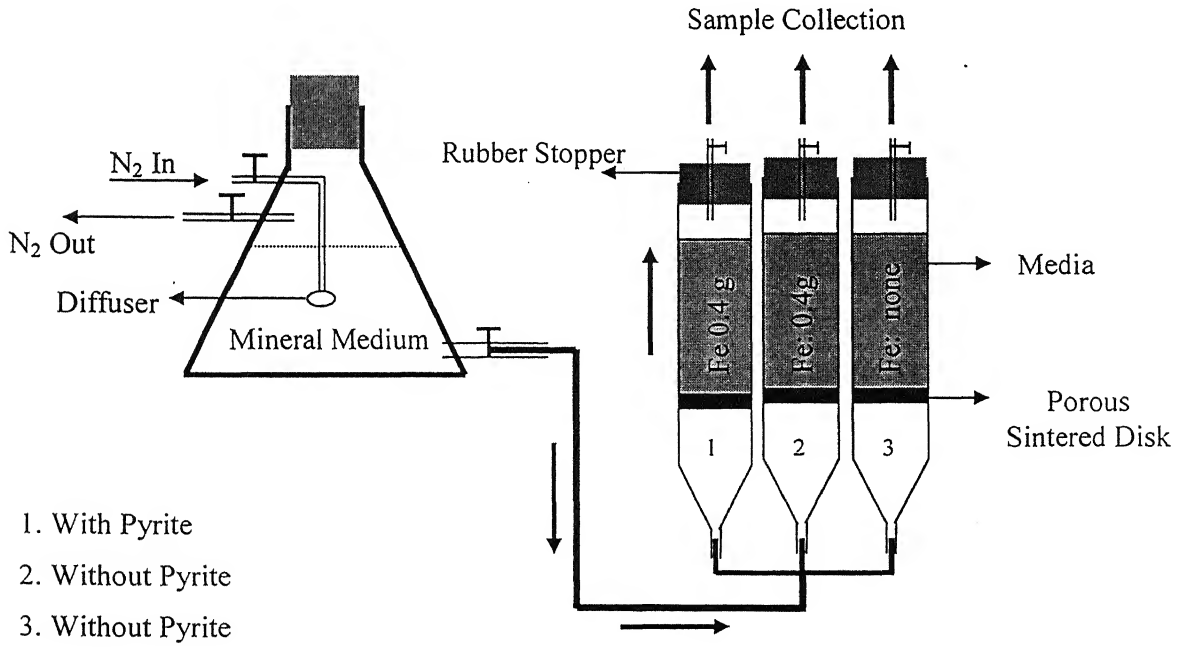
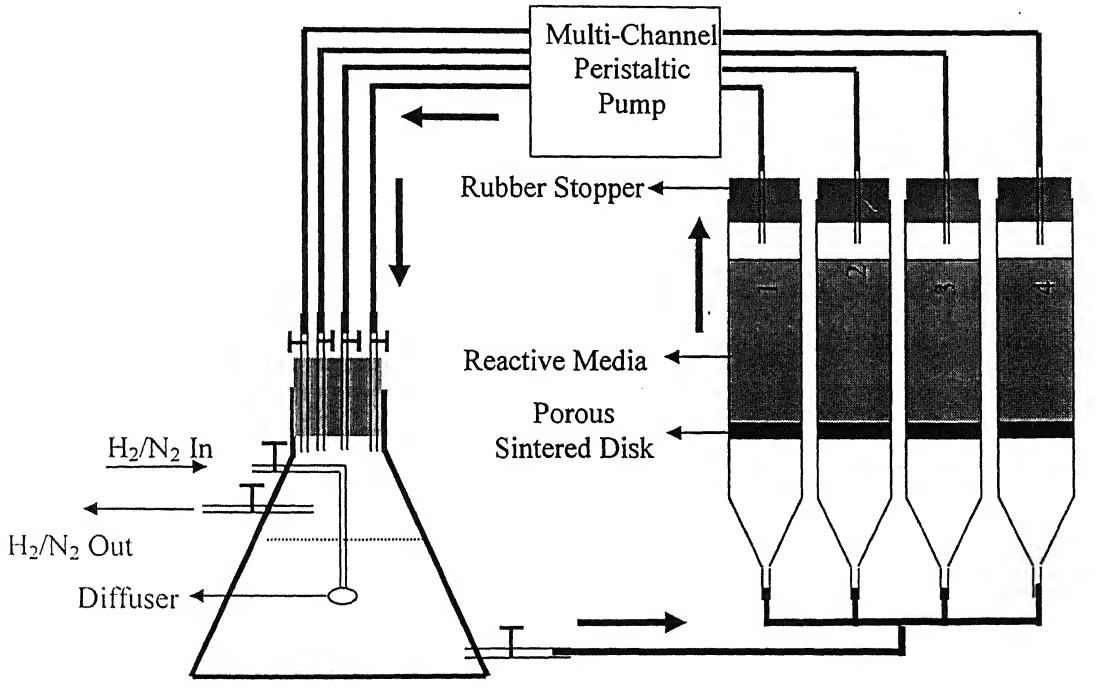


Figure 4.3: Schematic of the Experimental Apparatus used for Investigating Abiotic Nitrate Reduction in Flow-Through Columns

controlling water flow out of the column were closed, and the apparatus maintained in sealed condition. Once every 24 hours, the closed valves, as described above, were opened and a fixed amount of effluent, either 10, 5 or 2.5 mL corresponding to retention times of 6.5, 13 or 26 days, was allowed to pass through the columns and collected under nitrogen pressure. The collected samples were sterilized again, and stored for measurement of residual $NO_3^- - N$, $NO_2^- - N$, $NH_3 - N$, sulfate and pH.

In case of experiments involving biological denitrification, up-flow columns similar to those used for abiotic experiments were employed. However, such columns had to be seeded with microorganisms before the commencement of the biological denitrification experiment experiment. The schematic of the apparatus used for seeding these columns is shown in Figure 4.4.



1. With Pyrite, 0.25g steel wool
2. Without Pyrite, 0.25 g steel wool
3. With Pyrite, 0.40 g steel wool
4. Without Pyrite, 0.40 g steel wool

Figure 4.4 Apparatus for Seeding Up-Flow Columns with Autotrophic Denitrifying Microorganisms

Four column experiments were carried out simultaneously, two each with steel wool concentrations of 0.25g and 0.40g respectively. 1 g of pyrite was mixed with the media in one column at each iron concentration. As in the case of abiotic experiments, the columns in this case were also in flooded and maintained in sealed condition for some time to ensure the onset of anoxic conditions. Then the columns were connected to the stock culture reservoir as shown in Figure 4.4. A multi-channel peristaltic pump (IKEA, Germany) was employed for re-circulating the stock culture through the column. Initially the stock culture was periodically purged with hydrogen from an external source. However after some time, this supply of hydrogen was stopped. Colonization of the sand media in the columns by microorganisms was considered to have occurred once despite stopping external hydrogen supply, nitrate reduction was observed in the system. To further demonstrate this point, a reactor containing sterilized mineral media with 40 mg/L of nitrate (as N) was purged with nitrogen gas and attached to the columns in place of the stock culture reactor. It was observed that continued re-circulation of nitrate-containing mineral media through the reactor resulted in reduction in nitrate concentration in the system, even when no external hydrogen was supplied.

After seeding, the actual biological denitrification experiments were carried out in an experimental setup similar to one shown in Figure 4.3. At the start of the biological denitrification experiments, the columns were connected to a reservoir containing a sterilized mineral medium prepared with IIT Kanpur ground water containing approximately 40 mg/L of nitrate (as N). Each column was then flushed with approximately 100 mL of the nitrate-containing mineral medium from the reservoir, so that the nitrate concentrations in the effluent from the columns were approximately the same as the initial nitrate concentration in the reservoir. After this, the valves connecting the reservoir to the columns and those controlling water flow out of the column were closed, and the apparatus maintained in sealed condition. Then as in the case of abiotic experiments, the closed valves were opened and a fixed amount of effluent was allowed to pass through the column and collected each day. The collected samples were sterilized, and stored for measurement of residual $NO_3^- - N$, $NO_2^- - N$, $NH_3 - N$, pH and sulfate.

CHAPTER 5
RESULTS AND DISCUSSION

5.1 Introductory Remarks

In the hydrogenotrophic denitrification process, nitrate is the electron acceptor, which is ultimately reduced to nitrogen, thus resulting in denitrification. The denitrification process as described above, leads to the formation of a number of intermediate-products, and ultimately molecular nitrogen, as depicted below.

$$\overset{+5}{N}O_3^- \rightarrow \overset{+3}{N}O_2^- \rightarrow \overset{+2}{N}O(g) \rightarrow \overset{+1}{N}_2O(g) \rightarrow \overset{0}{N}_2(g) \qquad (5.1)$$

A mixed culture of hydrogenotrophic anaerobic denitrifying microorganisms capable of effecting denitrification as above was developed and maintained, and used for the research described here. Hydrogen required for the sustenance of above denitrification process was generated 'in-situ', through anaerobic corrosion of metallic iron in aqueous medium. This is achieved by adding metallic iron to a system containing nitrate contaminated aqueous medium and the denitrifying microorganisms. Thus the overall reaction for metallic iron-assisted hydrogenotrophic denitrification is as below,

$$(Fe(0) + 2H_2O \rightarrow H_2 + Fe^{2+} + 2OH^-) \times 5 \qquad (5.2a)$$

$$2NO_3^- + 5H_2 \rightarrow N_2 + 4H_2O + 2OH^- \qquad (5.2b)$$

$$5Fe(0) + 2NO_3^- + 6H_2O \rightarrow 5Fe^{2+} + N_2 + 12OH^- \qquad (5.2)$$

However, in the same system, simultaneous abiotic nitrate reduction to ammonia is also possible, as depicted below,

$$\overset{+5}{N}O_3^- \rightarrow \overset{+3}{N}O_2^- \rightarrow \overset{-3}{N}H_4^+ \qquad (5.3)$$

The overall equation for metallic iron-assisted abiotic nitrate reduction is as below,

$$4Fe(0) + NO_3^- + 7H_2O \rightarrow 4Fe^{2+} + NH_4^+ + 10OH^- \qquad (5.4)$$

Since ammonia formation as a result of abiotic nitrate reduction is undesirable, it is important to control the type and quantity of metallic iron to be added for hydrogenotrophic denitrification such that ammonia formation due to abiotic nitrate

reduction is minimized, while maintaining a reasonable rate of biological denitrification sustained by hydrogen production through metallic iron corrosion.

Additionally, both biological and abiotic nitrate reduction reactions (Equations 5.3 and 5.4) result in the production of OH^- ions, which may increase the pH of poorly buffered systems. This pH increase will inhibit the microbial activity when the system pH becomes greater than 9. Hence, for sustenance of long-term hydrogenotrophic denitrification, adequate buffering must be provided such that pH increase is not excessive and detrimental to the nitrate reduction.

Based on results of research conducted previously (Biswas 2002) in our laboratory, it was concluded that of different types of metallic iron tested, commercially available steel wool with its relatively low specific surface area was quite suitable for stimulating hydrogenotrophic denitrification, while having the lowest propensity to abiotically reduce nitrate to ammonia. Further research carried out in our laboratory (Lavania, 2003) showed that passing of nitrate contaminated water through up-flow columns containing 125 cm^3 of sand mixed with 0.25 g steel wool, and seeded with hydrogenotrophic denitrifying microorganisms resulted in nitrate concentration being reduced from 40 mg/L (as N) to around 5 mg/L (as N), while ammonia formation was only 2 mg/L (as N). The retention time of water in such columns was 26 days.

5.2 Buffering During Hydrogenotrophic Denitrification

Hydrogenotrophic denitrification as described above results in the production of hydroxide ions, which in the absence of suitable buffering may increase the pH of the system and hamper microbial activity at pH greater than 9 (Till et al., 1998). Biswas (2002) and Lavania (2003) circumvented this problem of pH increase by adding large quantities of phosphate buffer to the nitrate containing samples being denitrified during their experiments. However, in practical situations natural water samples may not have sufficient buffering capacity to resist pH increase. Under such circumstances, the best option is to fortify the reactive media in which the denitrification is taking place with

suitable buffering agents. Considering the long-term buffering action that is required for sustenance of continuous flow systems, the buffering agents required for this purpose must be solid state, which will dissolve slowly due to perturbations in water chemistry caused by denitrifying reactions, and thus provide the required buffering. Lavania (2003) recommended the use of the mineral Pyrite (FeS_2) for this purpose.

5.2.1 Theoretical Basis of Buffering Action By Pyrite

Reactions involving iron pyrite (FeS_2) present as a mineral in an aqueous system are shown in Table 5.1. Pyrite is stable under severely reducing conditions. Under less severe reducing conditions, it is only stable at low pH values, and tends to get oxidized at higher pH values as per Equation Nos. 11 and 13 given in Table 5.1, with sulfate being formed as the oxidation product. The ferrous ions released due to this oxidation process consume hydroxide ions and are converted to $Fe(OH)_2$ precipitate as per Equation No. 12 in Table 5.1. Pyrite is however, unstable even under moderately reducing conditions, being readily oxidized to sulfate and $Fe(OH)_2$ (s).

Pyrite dissolution and consequent formation and co-existence of $Fe(OH)_2$ solid phase with pyrite, provides buffering to the aquatic system. In a system where hydroxide ions are constantly added, as is the case during hydrogenotrophic denitrification, the system pH is expected to rise if little or no buffering is present. However, in presence of iron pyrite, the hydroxide ions added will be consumed and pyrite dissolved as per Equation 5.5 (Based on Equations 11, 12 and 13) in Table 5.1,

$$FeS_2 + 18.OH^- \rightarrow Fe(OH)_2 \downarrow + 2.SO_4^{2-} + 14.e + 8.H_2O \qquad (5.5)$$

The pH in that case will remain relatively constant through the buffering action of pyrite dissolution.

To further elucidate the buffering action equations given in Table 5.1 were evaluated using the chemical speciation software MINEQL+, and pC-pH diagram computed at pE value of –3 for a system containing 0.5 mM pyrite, initially in solid phase.

Table 5.1 Relevant Equations Responsible for the Buffering Action by Pyrite

Equation Number	Equation	Equilibrium Constant
1.	$H_2O \leftrightarrows H^+ + OH^-$	$K_1 = 10^{-14.000}$
2.	$3.H_2O + Fe^{2+} \leftrightarrows Fe(OH)_3^- + 3.H^+$	$K_2 = 10^{-31.000}$
3.	$2.H_2O + Fe^{2+} \leftrightarrows Fe(OH)_2(aq) + 2.H^+$	$K_3 = 10^{-20.570}$
4.	$H_2O + Fe^{2+} \leftrightarrows Fe(OH)^+ + H^+$	$K_4 = 10^{-9.500}$
5.	$H^+ + SO_4^{2-} \leftrightarrows HSO_4^-$	$K_5 = 10^{1.987}$
6.	$HS^- \leftrightarrows HS^- + H^+$	$K_6 = 10^{-12.918}$
7.	$HS^- + H^+ \leftrightarrows H_2S(aq)$	$K_7 = 10^{6.994}$
8.	$Fe^{2+} + SO_4^{2-} \leftrightarrows FeSO_4(aq)$	$K_8 = 10^{2.250}$
9.	$Fe^{2+} + 2.HS^- \leftrightarrows Fe(HS)_2(aq)$	$K_9 = 10^{8.950}$
10.	$Fe^{2+} + 3.HS^- \leftrightarrows Fe(HS)_3^-$	$K_{10} = 10^{10.987}$
11.	$2.HS^- + Fe^{2+} \leftrightarrows FeS_2$ (Pyrite)$\downarrow + 2.H^+ + 2.e$	$K_{11} = 10^{18.479}$
12.	$2.H_2O + Fe^{2+} \leftrightarrows Fe(OH)_2 \downarrow + 2.H^+$	$K_{12} = 10^{-12.100}$
13.	$8.e + 9.H^+ + SO_4^{2-} \leftrightarrows 4.H_2O + HS^-$	$K_{13} = 10^{33.660}$

Perusal of Figure 5.1A shows that at a pE value of –3, and pH approximately between 7.38 and 7.78, pyrite is converted to $Fe(OH)_2$. Corresponding theoretically calculated titration and buffer intensity curves are shown in Figure 5.1B and 5.1C respectively. It can be concluded from these curves that large amount of hydroxide ions are required to increase pH from 7.38 to 7.78 in such a system, with highest buffer intensity being 24.8 mM/unit pH at pH 7.76.

It must however be realized that the calculations described above are for equilibrium conditions, and the efficiency of the above buffering action depends on the kinetics of

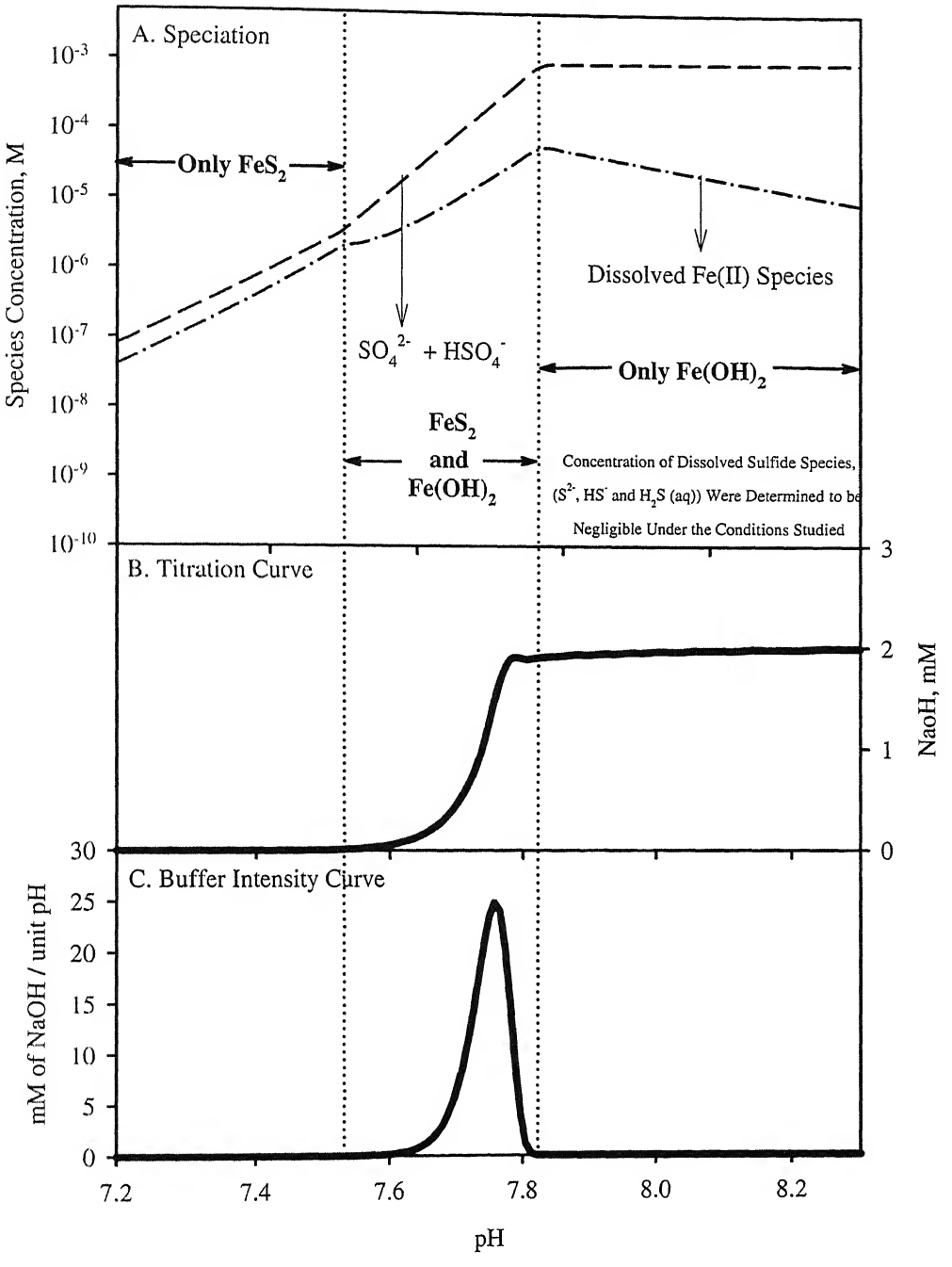


Figure 5.1 Calculation of Equilibrium Speciation in a Solution Containing 0.5 mM concentration of Solid Iron Pyrite (FeS_2) and the Corresponding Buffer Intensity Indicating Resistance to pH Increase

(Sample pE = -3; Calculations Performed Using MINEQL, a Chemical Speciation Software)

pyrite dissolution and $Fe(OH)_2$ formation, which will be experimentally evaluated in the next section.

5.2.2 Preliminary Experimental Verification of Buffering Action By Pyrite

The purpose of the preliminary experiments described here was to experimentally verify the buffering action of pyrite. For this purpose, 1 g of pyrite was added to 300 mL de-oxygenated IIT Kanpur groundwater with the following characteristics; pH 7.46, alkalinity 250 mg/L (as $CaCO_3$) and sulfate concentration of 10.5 mg/L, spiked with 40 mg/L of nitrate (as N). Another sample was prepared in exactly similar manner but with no pyrite addition. NaOH was added to both samples daily. The pH change in these samples was as shown in Figure 5.2. Based on these results it was concluded that pyrite was effective in arresting pH increase in a system where NaOH was constantly being added.

Sulfate concentration in the system containing pyrite increased from and initial value of 10.5 mg/L to 95 mg/L at the end of the experiment. Based on this evidence, it is postulated that the buffering provided was through pyrite dissolution, as described in the previous section. Pyrite oxidation in this case is abiotic, since samples were sterilized before the experiment. However, the identity of the species acting as electron acceptor during pyrite oxidation is not known with certainty. It is unlikely that oxygen is the electron acceptor, since the sample was de-oxygenated prior to the experiment. It is however possible that various ferric oxy-hydroxide, Fe(III), species, either present in the IITK groundwater or present as impurities in the pyrite added to the sample may have acted as the electron acceptor. Literature reports indicate that abiotic oxidation of pyrite by Fe(III) compounds under anaerobic conditions is quite rapid (Moses *et al.*, 1987).

5.3 Experiment Type I

These experiments were designed to determine the efficiency of pyrite in buffering systems where hydrogenotrophic denitrification was occurring, and also to determine the effect of pH increase on such denitrification rate. Experimental procedure involved

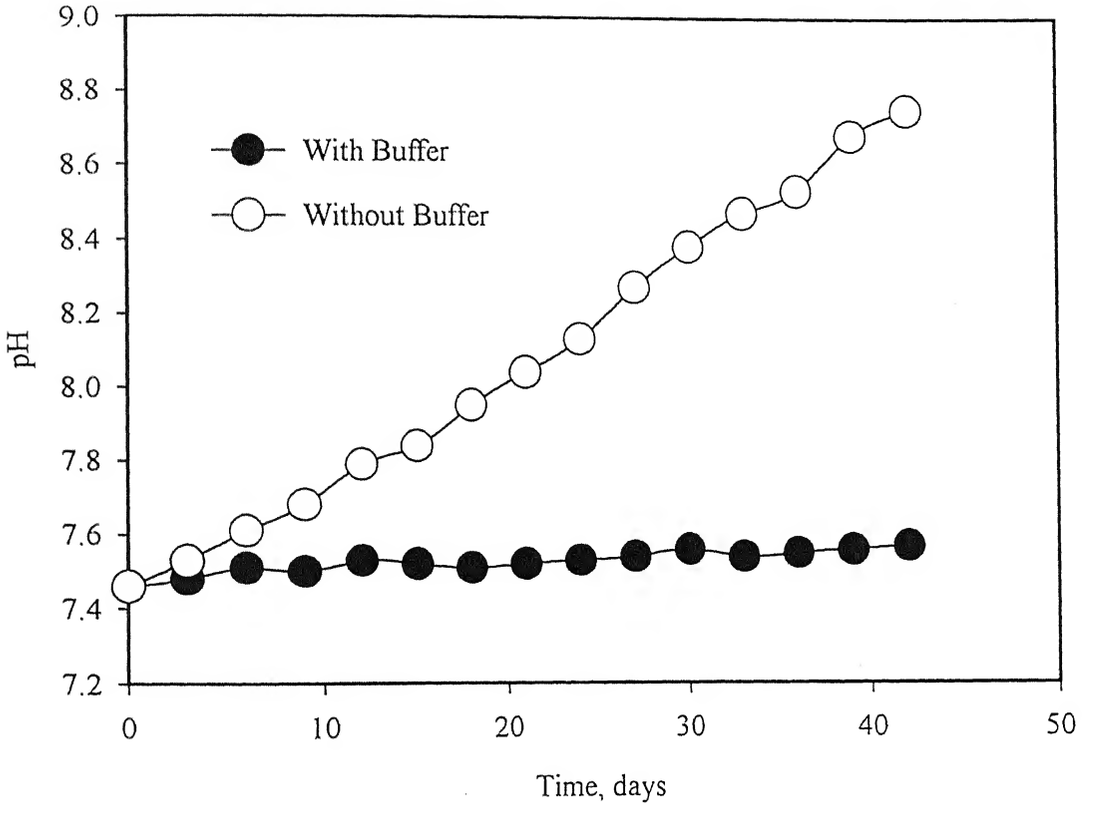


Figure 5.2 Preliminary Experiment Demonstrating Buffering Efficiency of Pyrite
Pyrite Added: 1 g in 300 mL of IIT K Groundwater;
NaOH Added: 10 mg (approx)/d

seeding bottles with varying nitrate concentrations viz., 40, 80, 120, and 240 mg/L nitrate (as N) with the autotrophic denitrifying microorganisms and maintaining conditions rich in hydrogen through external hydrogen supply. Experiments were carried out at each nitrate concentration with and without pyrite. Evolution of nitrate, nitrite, ammonia, and pH variation was monitored with time in all reactors to determine the extent of denitrification. Results of these experiments are shown in Figure 5.3.

As shown in Figure 5.3E, when pyrite not present, pH in reactors with 40, 80, 120 and 240 mg/L nitrate (as N) increased from the initial value of 7.56 to 9.13, 9.63, 9.79 and 10.02 respectively in the reaction period of 45 days. In the presence of pyrite as a buffering agent, the pH in similar reactors increased from an initial value of 7.56 to 7.85, 8.01, 8.05 and 8.21 respectively in the same reaction period. This proved that pyrite was effecting in arresting the pH increase in the reactors over the experimental duration of 45 days.

Comparative assessment of the extent of reduction in nitrate concentration in un-buffered and buffered reactors with initial nitrate concentration of 40, 80, 120 and 240 mg.L (as N) are shown in Figures 5.3A-D respectively. In all cases, at the end of the 45-day reaction period, the extent of nitrate reduction in buffered reactors was more than in their un-buffered counterparts. This difference is mainly due to a precipitous decline in the rate of nitrate reduction in the un-buffered reactors when the pH increases to 9 or above. Nitrite was observed as a by-product of biological nitrification in all reactors. Nitrite concentration in all reactors was around 2 mg/l (as N) after the 45-day reaction period. No ammonia formation was noticed in any reactor, signifying the lack of abiotic nitrate reduction through interaction with pyrite or other mechanisms.

5.4 Experiment Type II

These experiments were designed to determine the buffering efficiency of pyrite during long-term metallic-iron assisted biological denitrification in batch reactors containing varying amounts of steel wool and seeded with hydrogenotrophic denitrifying

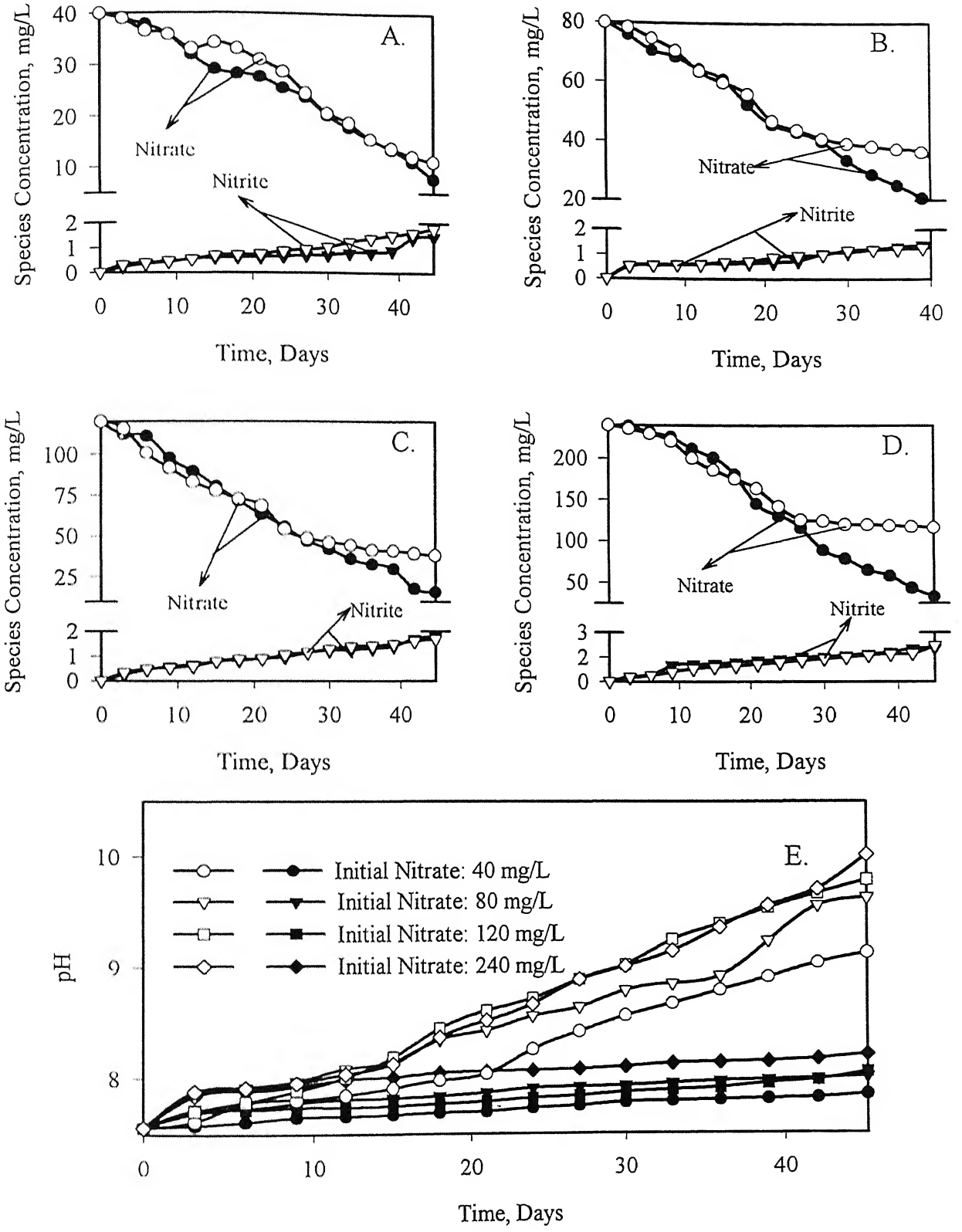


Figure 5.3 Autotrophic Denitrification in Hydrogen-Fed Reactors Containing Various Nitrate Concentrations in the Presence and Absence of Iron Pyrite as Buffer
(White Symbols: Unbuffered Reactors; Black Symbols: Buffered Reactors)

microorganisms. For comparison, reactors prepared in similar way, but containing no microorganisms was also evaluated.

Results of batch experiments where 0.1 g steel wool was added are presented in Figure 5.4. For both abiotic experiments, i.e., un-buffered case (Figure 5.4A) and the buffered case (Figure 5.4C), the total nitrogen concentration, i.e., sum of nitrate, nitrite and ammonia concentrations, did not decline over the reaction period of 75 days. This is expected because no denitrification is expected in these reactors in the absence of microorganisms. Nitrate concentration however declined in both reactors, as nitrate was reduced abiotically to nitrite and ammonia. Nitrite concentration was observed to increase with time before declining, suggesting that it is an intermediate product in the abiotic nitrate reduction process. Ammonia concentration however, increased with time, suggesting that it is the end product of the abiotic nitrate reduction process. In the un-buffered reactor, pH increased from 7.55 to 9.14, while pH in the buffered reactor increased from 7.55 to 7.73 over the reaction period (see Figure 5.4E). This suggests that pyrite was effective in providing buffering in the abiotic reactor, thus arresting pH increase during nitrate reduction. The extent of decline in nitrate concentration and corresponding increase in ammonia concentration was roughly same in both reactors, suggesting that neither the pH increase in the unbuffered reactor, nor the presence of pyrite in the buffered reactor impacted abiotic nitrate reduction.

For both biological experiments, i.e., un-buffered case (Figure 5.4B) and the buffered case (Figure 5.4D), the total nitrogen concentration, i.e., sum of nitrate, nitrite and ammonia concentrations, declined over the reaction period of 75 days, though the extent of denitrification was more in the buffered reactor. In the un-buffered reactor, pH increased from 7.51 to 9.57, while pH in the buffered reactor increased from 7.51 to 7.72 over the reaction period (see Figure 5.4E). This suggests that pyrite was effective in providing buffering in the biological reactors also, where nitrate reduction occurs both through the biological and abiotic processes. In the unbuffered rector (Figure 5.4 B), decline in total nitrogen concentration due to biological denitrification stopped after

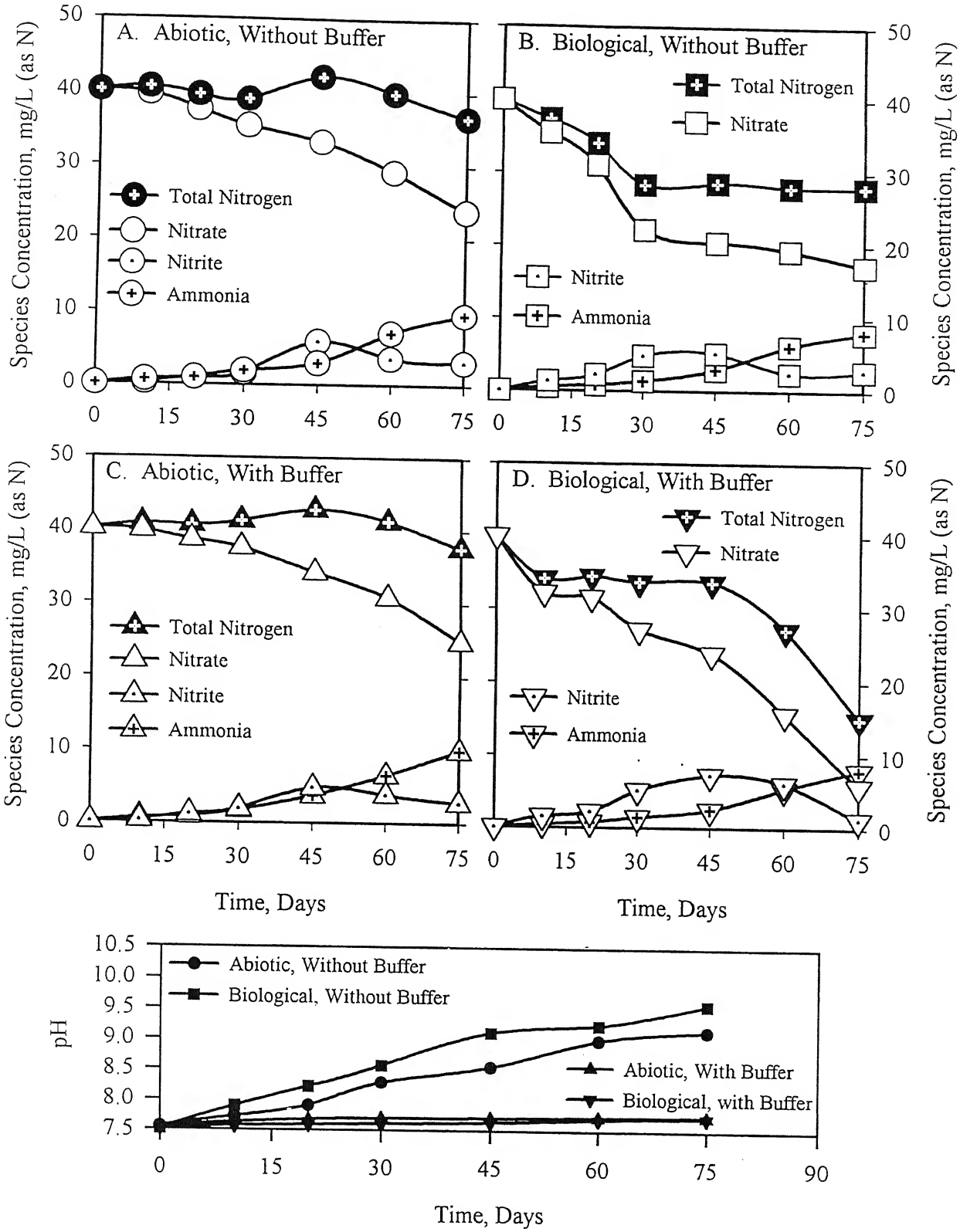


Figure 5.4 Abiotic and Biological Transformation of Nitrate in Batch Reactors in the Presence of 0.10 g Steel Wool
Reactor Volume: 300 mL; Buffer: 1 g pyrite/300 mL (Where Added).

approximately 30 days. This time roughly corresponded with the pH in this reactor approaching 9, at which point, as discussed in the previous section, biological nitrate reduction is expected to cease. Nitrate reduction however continued in the reactor beyond this point through abiotic means, as shown by decline in nitrate and nitrite concentration and increase in ammonia concentration in the reactor with time. In comparison, decline in total nitrogen concentration in the buffered reactor (Figure 5.4D) continued throughout the experimental period, as pH increase was controlled in this reactor.

Experiments similar to the ones described above were carried out in batch reactors with steel wool concentrations of 0.25g and 0.40 g. Results obtained from these experiments are presented in Figures 5.5 and 5.6 respectively. Conclusions similar to ones drawn from Figure 5.4 may also be drawn from results presented in Figures 5.5 and 5.6. Comparing the results presented in Figures 5.4, 5.5 and 5.6 one may draw certain additional conclusions regarding the impact of steel wool concentration on abiotic and biological nitrate reduction. First, during abiotic nitrate reduction, the extent of nitrate reduction and hence ammonia formation increases with increase in the steel wool concentration in the reactor. Second, during biological experiments in the buffered reactor, the extent of denitrification decreases slightly, ammonia formation increases slightly, and nitrate concentration remains nearly unchanged with increase in steel wool concentration in the reactor.

5.5 Experiment Type III

The objective of these experiments was to demonstrate the efficiency of pyrite in providing buffering during long-term hydorgenotrophic denitrification in reactive porous medium containing metallic iron and denitrifying microorganisms. As per recommendations from an earlier study (Lavania, 2003), two types of reactive media were prepared, by mixing 125 cm^3 of sterilized, acid washed sand, 1 g of pyrite and 0.25, or 0.40 g of steel wool. Experimental details have been mentioned earlier.

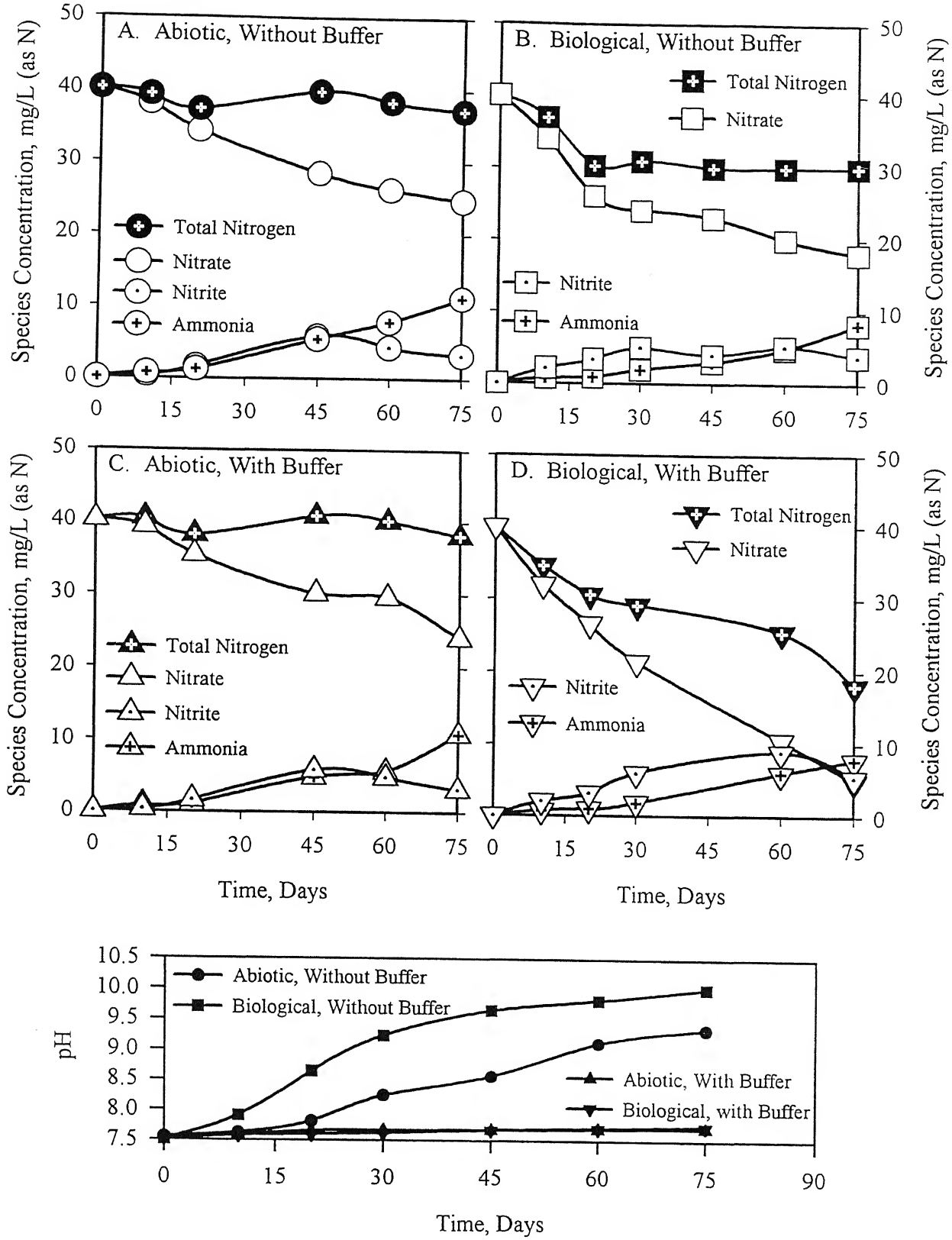


Figure 5.5 Abiotic and Biological Transformation of Nitrate in Batch Reactors in the Presence of 0.25 g Steel Wool
Reactor Volume: 300 mL; Buffer: 1 g pyrite/300 mL (Where Added).

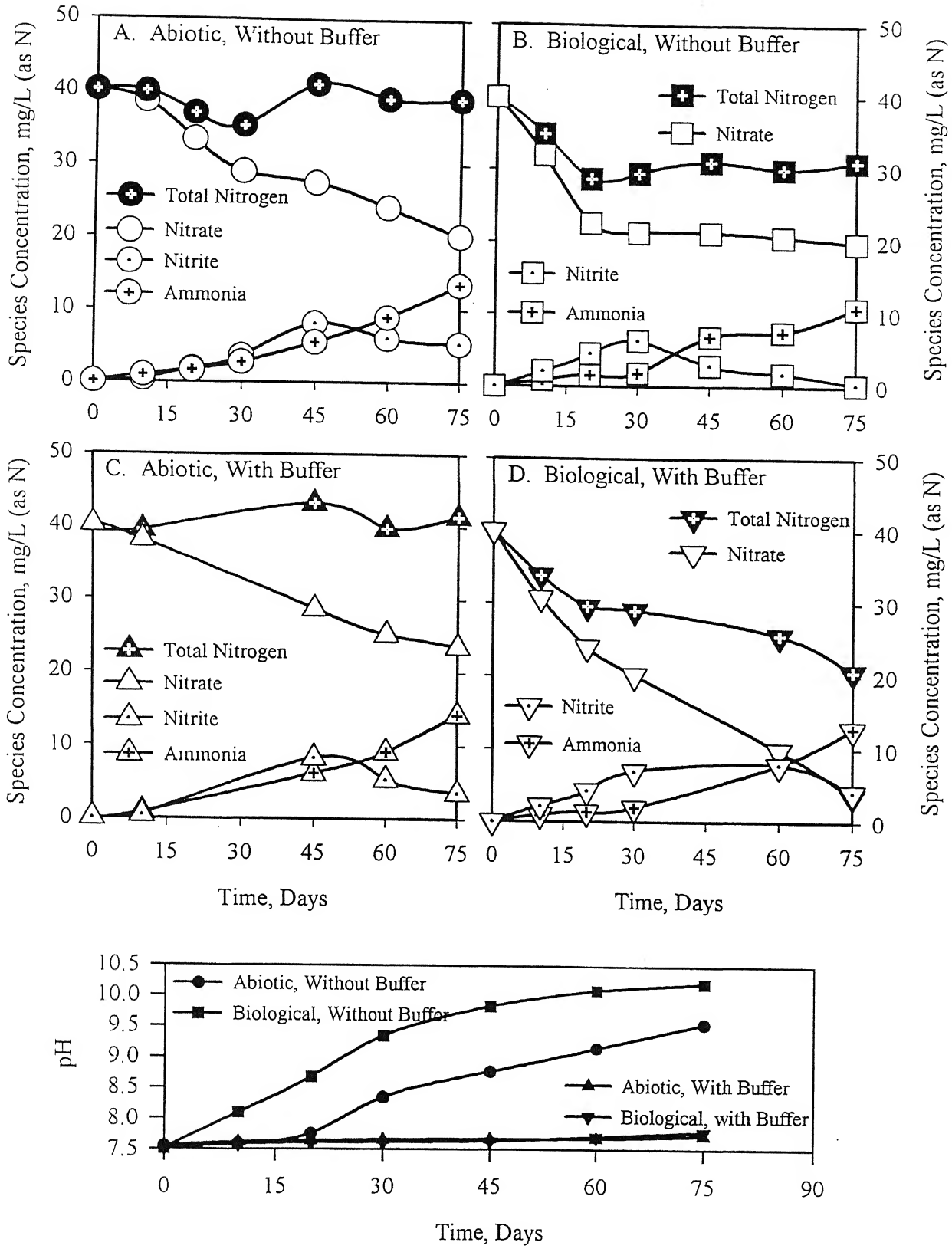


Figure 5.6 Abiotic and Biological Transformation of Nitrate in Batch Reactors in the Presence of 0.40 g Steel Wool
Reactor Volume: 300 mL; Buffer: 1 g pyrite/300 mL (Where Added).

Three experiments were carried out using unseeded columns maintained in sterile conditions by addition of $HgCl_2$. One of these columns contained only sand, the other sand and 0.4 g of 'steel-wool', while the third one contained sand, 0.4 g steel wool and 1 g pyrite. The influent nitrate concentrations in all columns were approximately 40 mg/L (as N). These columns were operated in up-flow mode, and a predetermined volume of water was withdrawn from the columns each day, thus controlling the retention time of water in the columns. Initial rate of effluent extraction from the columns corresponded to a retention time of 6.5 days. The retention time was progressively increased to 13 days and then to 26 days by progressively decreasing the rate of effluent extraction. Effluent nitrate, nitrite and ammonia concentrations from the columns were measured every two days. Results of the experiment conducted with the reactor containing only sand is shown in Figure 5.7. The effluent nitrate concentration was found to be nearly unchanged at 40 mg/L over the duration of the experiment. Ammonia and nitrate concentrations in the effluent were generally less than 1 mg/L. These results are as per expectations, since no obvious nitrate reduction and hence ammonia formation mechanism existed in this column. In case of similar experiments carried out in sterile columns containing sand and steel wool (Figures 5.8A), and that containing sand, steel wool and pyrite (Figure 5.8B), ammonia concentration in the effluent was much higher than in the column with only sand (Figure 5.7) for all retention times. This is consistent with abiotic nitrate reduction to ammonia in presence of metallic iron in abiotic conditions. It was also observed that ammonia and nitrite concentration in the effluent from this column showed a general upward trend with increase in retention time of water inside the column. This is also easily explicable, considering that more retention time affords more opportunity for abiotic nitrate reduction. Comparing results presented in Figures 5.8A and 5.8B it is noted that presence of pyrite in the latter column had no discernable effect on the rate of nitrate reduction or ammonia formation. However, pH of water effluent from the columns without and with buffering, at the end of the 30 day reaction period, were 8.68 and 7.63 respectively, while the pH of influent water in both

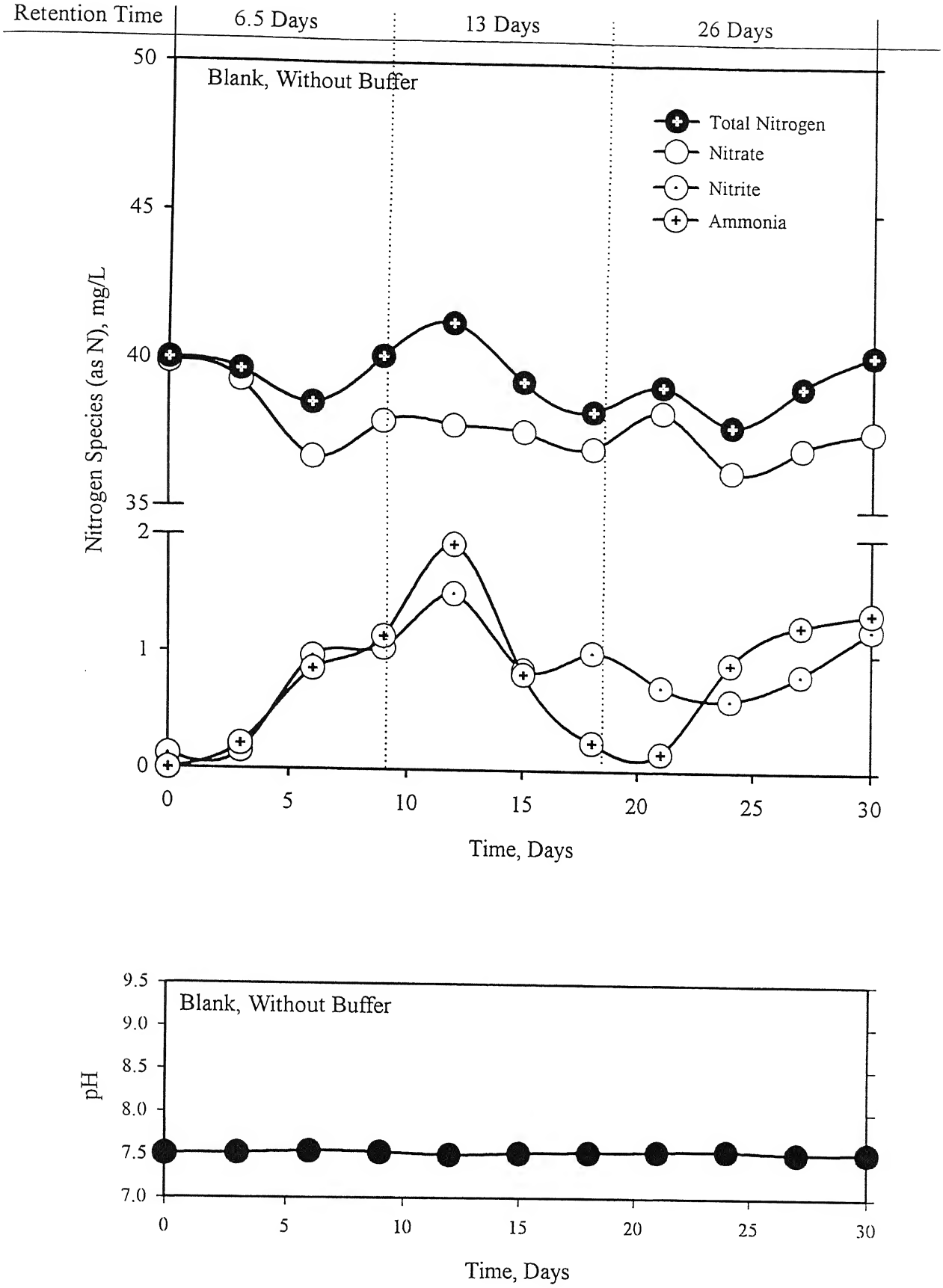


Figure 5.7 Evolution of Nitrate in an Intermittent-Flow Upflow Column Reactor Containing No Denitryfying Microorganisms or Metallic Iron (Volume of Sand: 125 cm^3)

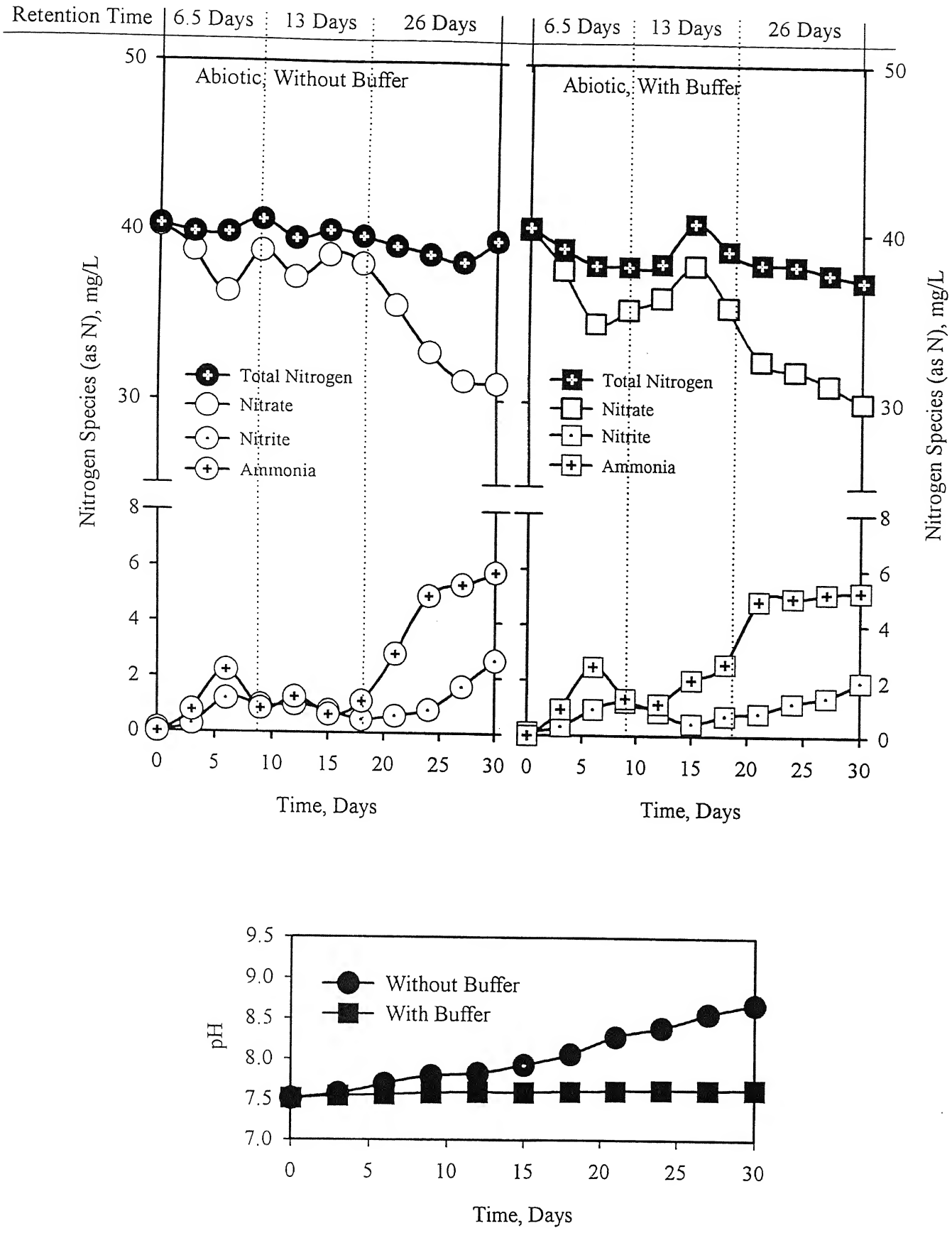
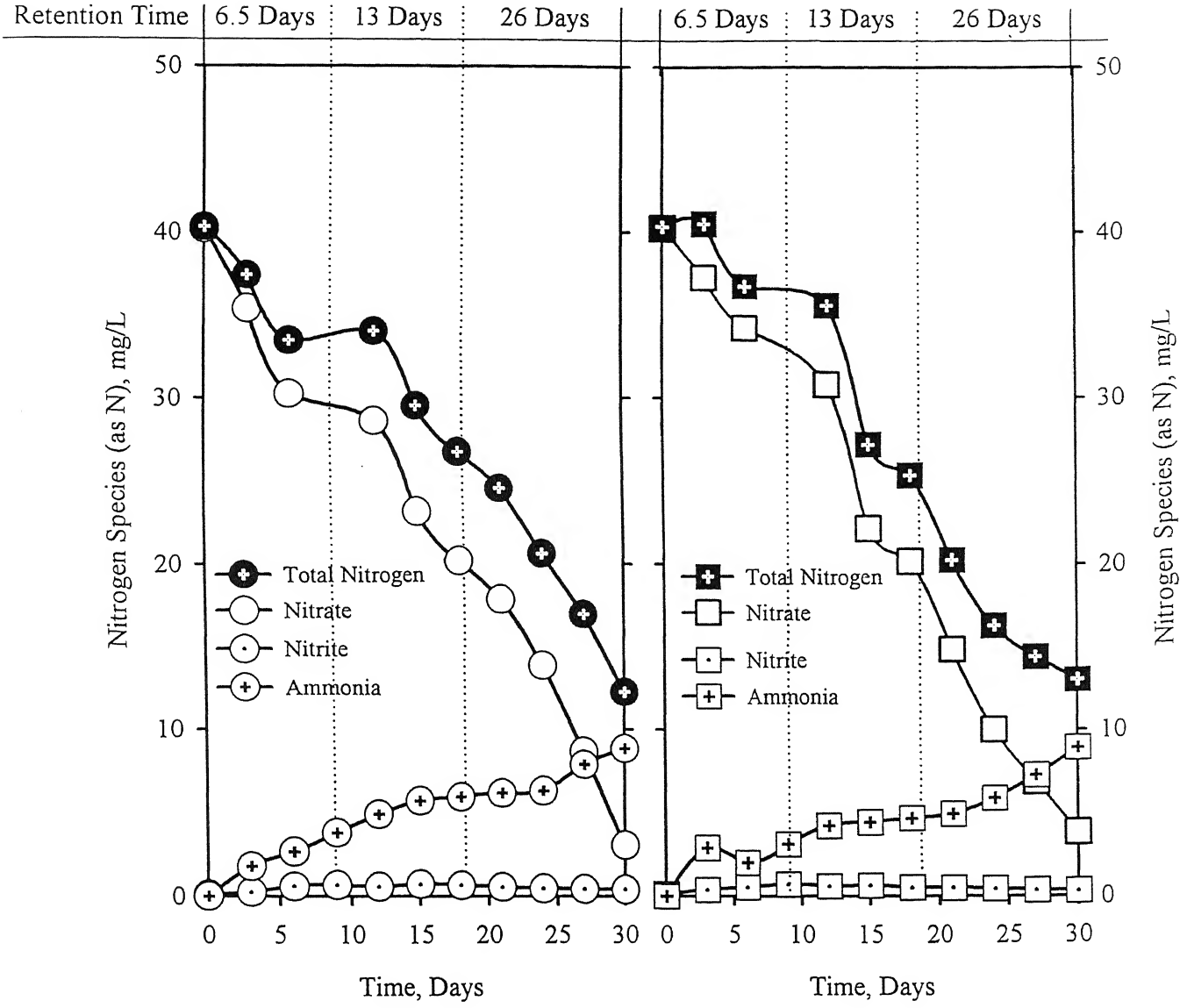


Figure 5.8 Abiotic Transformation of Nitrate in an Intermittent-Flow Upflow Column Reactor Containing Sand and 0.40 g of Metallic Iron
(Volume of Sand: 125 cm^3)

cases was 7.51 (Figure 5.8C). This suggests that pyrite was effective in arresting pH increase of water effluent from the buffered column.

Four experiments were carried out using columns seeded with autotrophic denitrifying microorganisms. The steel wool concentration in two of these columns was 0.25 g, while it was 0.40 g in the other two columns. Further, one column at each iron concentration was buffered with pyrite. The influent nitrate concentrations, mode of operation and sample collection techniques in these columns were similar to what was described earlier, in case of abiotic columns. Nitrate reduction results obtained from biological experiments carried out in columns containing 0.40 g of steel wool is presented in Figures 5.9A and 5.9B for the un-buffered and buffered cases respectively. The experimental period in both cases was 30 days, which included maintenance of the columns at a retention time of 6.5 days for 9 days, at a retention time of 13 days for the next 9 days and at a retention time of 26 days for the last 12 days. At the end of experimental period, the effluent nitrate concentration from both the columns had declined to around 3-4 mg/L, while the influent nitrate concentration was maintained constant 40 mg/L throughout the experimental duration. Effluent ammonia concentration in both cases had however increased to approximately 8-9 mg/L, and the effluent nitrite concentration was below 0.5 mg/L. In the un-buffered column, effluent pH increased with time and was finally at 9.62 at the end of the 30-day experimental period (Figure 5.9C), while the influent pH remained constant at 7.51 throught the experimental duration. In the buffered column, however, effluent pH at the end of the 30-day experimental period was only 7.76, suggesting that pyrite was effectively buffering the system against pH increase.

Nitrate reduction results obtained from biological experiments carried out in columns containing 0.25 g of steel wool is presented in Figures 5.10A and 5.10B for the un-buffered and buffered cases respectively. The experimental procedure and conditions in this case were similar to that described in the previous case. In these experiments, the effluent nitrate concentration from both the columns had declined to around 5 mg/L,



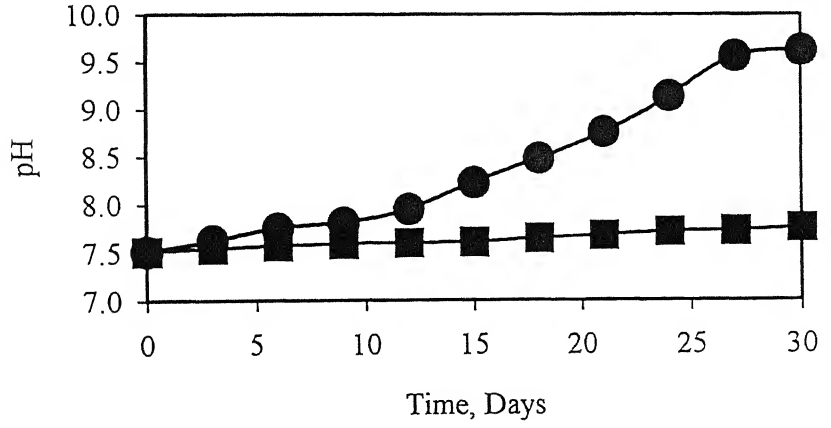
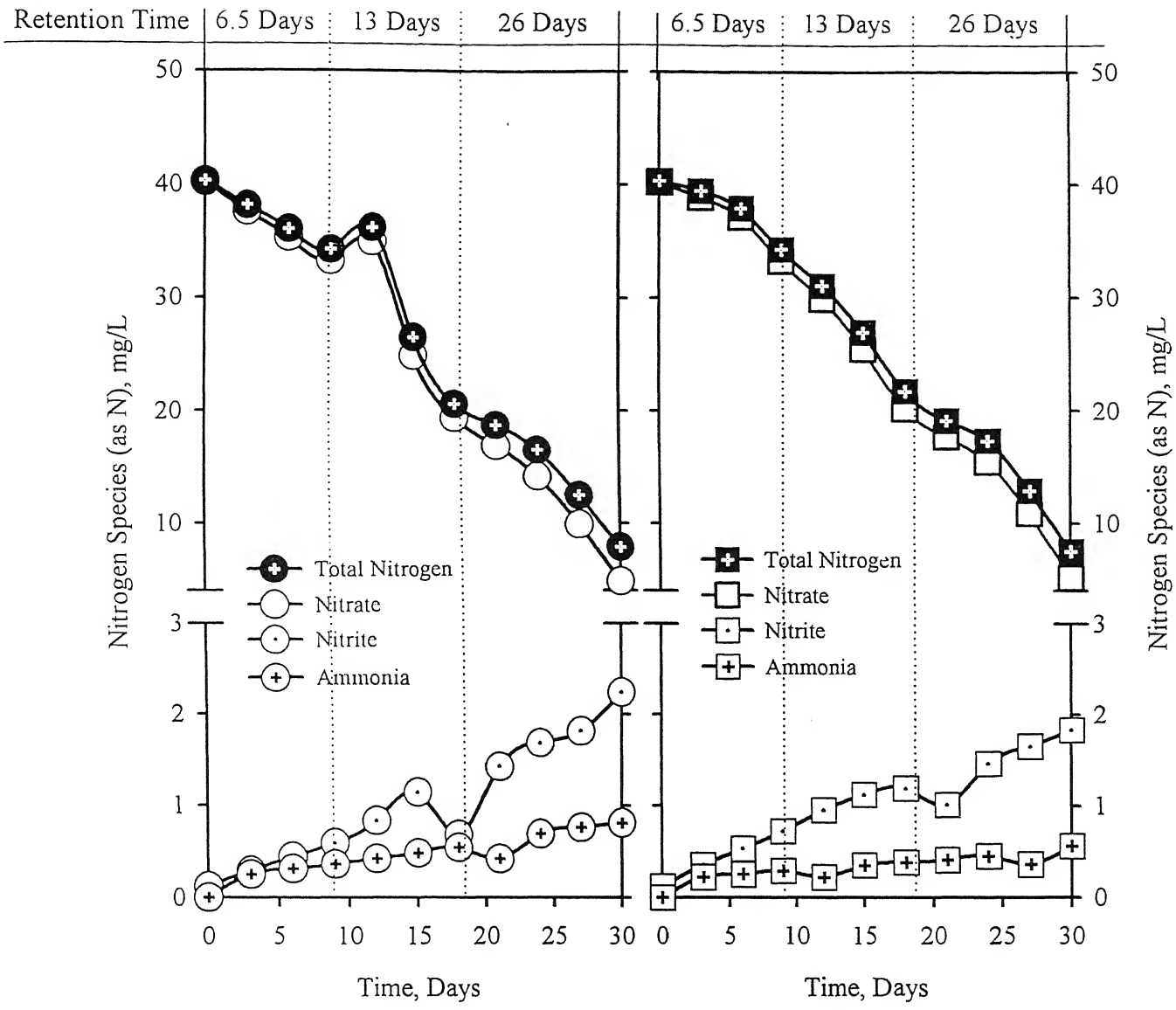


Figure 5.9 Biological Transformation of Nitrate in an Intermittent-Flow Upflow Column Reactor Containing Sand and 0.40 g of Metallic Iron
(Volume of Sand: 125 cm^3)



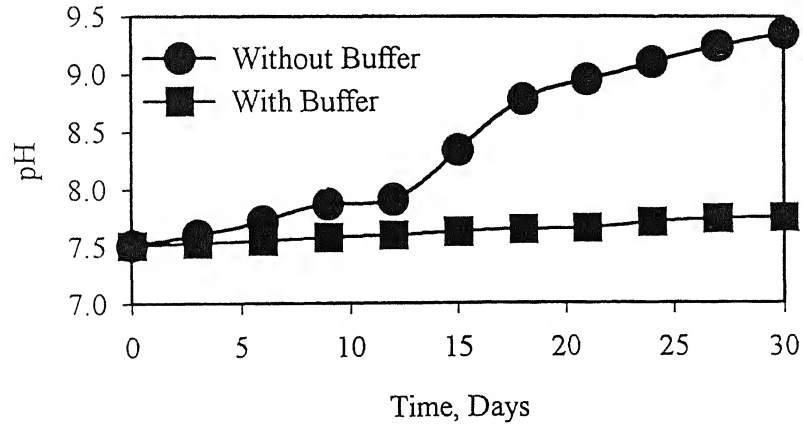


Figure 5.10 Biological Transformation of Nitrate in an Intermittent-Flow Upflow Column Reactor Containing Sand and 0.25 g of Metallic Iron (Volume of Sand: 125 cm^3)

while the influent nitrate concentration was 40 mg/L throughout the experimental duration. Effluent ammonia concentration in both cases had only increased to approximately 2 mg/L, and the effluent nitrite concentration was less than 1 mg/L. In the un-buffered column, effluent pH increased with time and was finally at 9.35 at the end of the 30-day experimental period (Figure 5.10C), while the influent pH remained constant at 7.51. In the buffered column, however, effluent pH at the end of the 30-day experimental period was only 7.75 suggesting that, in this case also, pyrite was effectively buffering the system against pH increase.

5.6 Discussions of Results

Hydrogenotrophic denitrifiers are anoxic microorganisms that thrive under moderately reducing conditions. Considerable pH increase is expected in poorly buffered flow-through systems, e.g., reactive porous media containing metallic iron, during such denitrification, because the hydrogenotrophic denitrification process produces hydroxide ions. Long-term pH control in such systems will require the provision of a solid buffering agent as an integral part of the porous media, which will neutralize the hydroxide ions generated during the denitrification process. This study investigated the efficiency of the buffering provided by the mineral pyrite due to its oxidative dissolution in presence of hydroxide ions.

Oxidative dissolution of pyrite requires the presence of a suitable electron acceptor in the system. In moderately reducing conditions, ferric oxy-hydroxide, trace dissolved oxygen or even dissolved organic carbon species may act as electron acceptor for pyrite dissolution. In fact, in the experiments involving pyrite reported in this study, oxidative pyrite dissolution and consequent sulfate formation was possible even under anoxic conditions maintained to facilitate biological denitrification.

One possible drawback of using pyrite as a buffering agent is the release of sulfate due to oxidative dissolution of pyrite. Measurements of sulfate concentrations in the batch reactors used for biological denitrification experiments (Figures 5.4 - 5.6) have shown

that removal of 1 g of nitrate results in the production of approximately 2 g of sulfate. However, sulfate is not a toxic substance, unless present in very high concentrations. Use of pyrite as a buffering agent does not appear to have a toxic effect on denitrifying microorganisms or increase the rate of ammonia formation through metallic iron-assisted abiotic denitrification.

The experiments reported in this study were carried out with a mixed culture of purely hyrogenotrophic denitrifying microorganisms. However, literature reports indicate the occurrence of sulfide-oxidizing dentrifiers (Barrenstein et al., 1986), e.g., *Thiobacillus denitrificans*, in many natural systems, which reduce nitrate as below,

$$5S^{2-} + 8NO_3^- + 8H^+ \rightarrow 4N_2 + 5SO_4^{2-} + 4H_2O \qquad (5.6)$$

Thus, in the presence of pyrite, it is entirely possible that sulfide-oxidizing denitrifiers may become active in systems designed for hydrogenotrophic denitrification. The impact of competition by these two species of autotrophic denitrifying microorganisms on overall biological denitrification rates is unknown. However, it all probability, hydrogenotrophic denitrifiers will be more active in moderately reducing conditions, and the sulfide-oxidizing denitrifiers in more severe reducing conditions.

CHAPTER VI
SUMMARY AND CONCLUSIONS

Metallic iron assisted autotrophic denitrification is feasible, but its practical application is limited by the requirement that abiotic reduction of nitrate to ammonia by metallic iron is minimized during biological denitrification, and that suitable buffering is provided to arrest pH increase during the denitrification process. Research is ongoing in our laboratory to overcome the above and other drawbacks of this process. Based on earlier research in our laboratory it was determined that commercially available 'steel-wool' showed the least propensity to abiotically reduce nitrate to ammonia. In experiments carried out to determine the extent of denitrification during flow through reactive porous media, i.e., sand, seeded with steel wool and denitrifying microorganisms, optimal media composition was determined to be 0.25 g steel wool per 125 cm^3 of sand. When water containing 40 mg/L nitrate (as N) was passed through this media at a retention time of 26 days, the effluent ammonia and nitrate concentrations were below 2 mg/L and 5 mg/L respectively. However considerable pH increase, and cessation of biological denitrification above pH 9 was observed in the reactive media during the denitrification process described above. One option for arresting pH increase may be to fortify the reactive media with additional buffering capacity. Considering the long-term buffering action required, it is obvious that the buffer chosen must be a solid material, which while being retained inside the reactive media will consume hydroxide ions formed during denitrification, and in the process provide the required buffering.

Hence, objective of the present study was to evaluate the suitability of pyrite (FeS_2) as a buffering agent for arresting pH increase during denitrification. Pyrite is considered promising for this purpose because it is a mineral which is unstable under moderately reducing, i.e., anoxic conditions, where it consumes hydroxide ions produced due to denitrification reactions and slowly gets oxidized to ferrous hydroxide $Fe(OH)_2$. Through the results of this study,

- The theoretical basis for the buffering action of pyrite during the denitrification process was established.

- It was demonstrated that pyrite was effective in controlling pH increase during denitrification in batch as well as flow through systems, with no detrimental effect on the denitrification process.

Measurement of iron concentration in the effluent from the reactive media buffered with pyrite indicated that dissolved iron concentration is rarely more that 2 mg/L, even when nitrogen removal by biological denitrification is 30 mg/l (as N) or higher. This suggested that dissolved iron released due to corrosion of metallic iron and pyrite dissolution was precipitating as amorphous $Fe(OH)_2$ inside the reactive media. Also, no dissolved sulfide was detected in the effluent during these experiments. Measurements however indicated elevated levels of sulfate in the effluent due to pyrite dissolution. But, under anoxic conditions, and at near-neutral pH values, pyrite dissolution rate is limited but the availability of suitable electron acceptor and a lack of hydroxide ions, and hence is likely to occur only as a response to hydroxide ions being added to the system due to denitrification. Another cause of concern is the precipitation of ferrous oxy-hydroxides and other precipitates and progressive growth of microbial biomass inside the reactive media, which may result in reduction of porosity of the reactive media, thus affecting the long-term efficiency of such media in regard to denitrification capacity. However, more information on this subject is required before anything more definitive can be said.

REFERENCES

APHA, WEF, AWWA, (1985). Standard Methods for Examination of Water and Wastewater. 16th Edition, APHA, Washington D.C., USA.

APHA, WEF, AWWA, (1995). Standard Methods for Examination of Water and Wastewater. 19th Edition, APHA, Washington D.C., USA.

Aslan, S. and Turkman, A. (2004) Simultaneous Biological Removal of Endosulfan ($\alpha+\beta$) and Nitrates from Drinking Waters Using Wheat Straw as Substrate. *Environment International*, 30, 4, 449-455.

Atlas, R.M. (1997) Principles of Microbiology. 2nd Edition, William C. Brown Publishers, Dubuque, Iowa, USA.

Babiker, I.A., Mohamed, M.A.A., Terao H., Kato, K., and Ohto, K. (2004). Assessment of Groundwater Contamination by Nitrate Leaching from Intensive Vegetable Cultivation Using Geographical Information System. *Environment International,* 29, 1009–1017.

Barrenstein, A., Kramer, U. and Obermann, P. (1986). Underground Treatment of Nitrate Rich Groundwater by Infiltration with Treated Wastewater or Methane-rich Natural Gas. *DVGW-Schriftenreihe, Wasser*, Frankfort, West Germany, 106, 99-116.

Batchelor, B. and Lawrence, A.W. (1978a). A Kinetic Model for Autotrophic Denitrification Using Elemental Sulfur. *Water Research*, 12, 12, 1075-1084.

Batchelor, B. and Lawrence, A.W. (1978b). Autotrophic Denitrification Using Elemental Sulfur. *J. Water Pollution Control Federation*, 50, 8, 1986-2001.

Benefield, L.D. and Randall, C.W. (1980). Biological Process Design for Wastewater Treatment, Prentice Hall, Inc., Englewood Cliffs, NJ 07632.

Biswas, S. (2002). Zero-Valent Iron, Fe (0)-Assisted Autotrophic Denitrification. M. Tech Thesis, *Indian Institute of Technology*, Kanpur, India.

Blecon, G., et al. (1983). Autotrophic Biological Denitrification by *Thiobacillus Denitrificans* On Sulfur-marl. *Revue Francaise des Sciences de l'Eau*, Paris, France, 2, 3, 267-279.

Bodik, I., Kratochvil, K., Gasparikova, E. and Hutnan, M. (2003). Nitrogen Removal in an Anaerobic Baffled Filter Reactor with Aerobic Post-treatment. *Bioresource Technology*, 86, 79-84.

Bouchard, D.C., Williams, M.K., and Surampalli, R.Y., (1992). Nitrate Contamination of Groundwater: Sources and Potential Health Effects. *Jour. of Am. Water Works Assoc.*, 84, 85-90.

Bullermann, M. and Keidel, T. (1986). Biological Denitrification of Groundwater and Surface Water. *Schriftenreihe-Institut fuer Wasserversorgung*, Darmstadt, West Germany, 25, 95-107.

Cervantes, F.J., Rosa, D.A.D. and Gómez J. (2001). Nitrogen Removal from Wastewaters at Low C/N Ratios With Ammonium and Acetate As Electron Donors. *Bioresource Technol.*, 79, 2, 165-170.

Challis, B.C., Outram, J.R. and Shuker, D.E. (1980). New Pathways for the Rapid Formation of N-nitrosamines Under Neutral and Alkaline Conditions. *IARC*, 31, 43-58.

Chang, C.C., Tseng, S.K. and Huang, H.K. (1999). Hydrogenotrophic Denitrification with Immobilized *Alcaligenes Eutrophus* for Drinking Water Treatment. *Bioresource Technology*, 69, 53-58.

Claus, G. and Kutzner, H.J. (1985a). Autotrophic Denitrification Thiobacillus Denitrificans. *Appl. Microb. and Biotechnol.*, 22, 289-296.

Claus, G. and Kutzner, H.J. (1985b). Physiology and Kinetics of Autotrophic Denitrification by *Thiobacillus denitrificans. Appl. Microb. and Biotechnol.*, 22, 283-288.

Comley, H. H. (1945). Cyanosis in Infant Caused by Nitrates in Well Water. *Jour. Am. Med. Assoc.*, 129, 112.

Dahab, M.F., and Woodbury, W.L. (1998). Biological Treatment Options for Nitrate Removal From Drinking Water. *Proceedings of the AWWA Inorganic Contaminants Workshop, San Antonio, Texas, USA*, Feb, 22-24.

Delvin, J.F., Eedy, R., and Butler, B.J. (2000). The Effects of Electron Donor and Granular Iron on Nitrate Transformation Rates in Sediments from a Municipal Water Supply Aquifer. *Jour. Cont. Hydrol.*, 46, 81-97.

Doyle, M.P., Beuchat, L.R., and Montville, T.J. (1997). Food Microbiology Fundamentals and Frontiers. ASM Press, Washington, DC, p786.

Edmunds, W.M., and Gaye, C.B., (1997). Naturally High Nitrate Concentrations in Groundwaters from the Sahel. *Jour. of Envir. Quality,* 20, 1231-1239.

Fan, A.M, and Steinberg, V.E. (1996). Health Implications of Nitrate and Nitrite in Drinking Water: An Update on Methemoglobinemia Occurrence and Reproductive and Developmental Toxicity. *Regul Toxicol Pharmacol* Sect, 23, 35- 43.

Fan, A.M, Willhite, C.C., and Book, S.A. (1987). Evaluation of the Nitrate Drinking Water Standard with Reference to Infant Methemoglobinemia and Potential Reproductive Toxicity. *Regul Toxicol Pharmacol* Sect., 7, 135–148.

Flere, J.M. and Zhang, T.C. (1999). Nitrate Removal with Sulfur-limestone Autotrophic Denitrification Processes. *Journal of Environmental Engineering*, 125, 8, 721-729.

Foster, S.S.D., Cripps, A.C., and Smith-Carington, A. (1982). Nitrate Leaching to Groundwater. *Philos. Trans. R. Soc. Lond.* 296, 477-489.

Frank, C. and Dott, W. (1985). Nitrate Removal from Drinking Water by Biological Denitrification. *Vom Wasser*, 65, 287-295.

Franke, O.L. and McClymonds, N.E. (1972). Summary of the Hydrologic Situation on Long Island, N. Y. as a Guide to Water-Management Alternatives, *U.S. Geol. Surv. Prof. Pap. 627-F*, http://pbisotopes.ess.sunysb.edu/reports/bleifuss.

Frick, B.R. and Richard, Y. (1985). Experience with Biclogical Denitrification in a Full Scale Drinking Water Treatment Plant. *Vom Wasser*, 64, 145-154.

Fuchs, U. (1985). Device and Method for Biological Denitrification of Water. Patent Application 3340549, Nov. 9, 1983, West Germany.

Gamble, T.N., Betlach, M.R. and Tiedje, J.M. (1976). Numerically Dominant Denitrifying Bacteria from World Soils. *Appl. Environ. Microbiol.*, 33, 4, 926-939.

Gangolli, S.D., Van der Brandt, P.A., and Feron, V.J. (1994) Nitrate, nitrite and N-nitrosocompounds. *Eur J Pharmacol,Environ Toxicol Pharmacol* Sect. , 292, 1–38.

Gayle, B.P., Boardman, G.D., Sherrard, J.H. and Benoit, R.E. (1989). Biological Denitrification of Water. *Journal of Environmental Engineering*, 115, 5, 930-943.

Goodrich, J.A., Lykins, B.W., Clark, R.M., (1991). Drinking Water from Contaminated Groundwater. *Journal of Environmental Quality, 20*, 707-717.

Hellekes, R. (1986). Nitrate Removal in Drinking Water Treatment by Using Autotrophic Microorganisms with Hydrogen Addition. *DVGW-Schriftenreihe, Wasser*, Frankfurt, West Germany, 106, 145-156.

Holzmacher, R.G., McLendon, S.C. and Murrell, N. E. (1970). Comprehensive Public Water Supply Study: Suffolk County, New York. Melville, New York: Holzmacher, McLendon and Murrel, Consulting Engineers, CPWS-24. v. 2, section 3-water resources.

Howard, K.W.F. (1985). Denitrification in a Major Limestone Aquifer. *Jour. Hydrol,* 76, 265-280.

Howarth, R., Anderson, D., Cloem, J., Elfring, C., Hopkinson, C., Lapointe, B., Malone, T., Marcus, N., McGlathery, K. Sharley, A., and Walker, D. (2000) Nutrient Pollution of Coastal Rivers, Bays and Seas. *Issues Ecol.* 7, 1-14 (Fall).

Hsieh, Y.L., Tseng, S.K., and Chang Y.J. (2003). Nitrogen Removal from Wastewater Using a Double-Biofilm Reactor with a Continuous-Flow Method. *Biosource Technol.*, 88, 107-113.

Hunter, W.J., (2003). Accumulation of Nitrite in Denitrifying Barriers when Phosphate is Limiting. *Jour. of Contaminant Hydrology*, 66, 79-91

Jun, B.H., Miyanaga, K., Tanji, Y., and Unno, H. (2003). Removal of Nitrogenous and Carbonaceous Substances by a Porous Carrier-Membrane Hybrid Process for wastewater treatment. *Biochem. Engg. Jour.*, 14, 37-44.

Kamolpornwijit, W., Liang L., West, O. R., Moline, G. R., and Sullivan, A. B., (2003). Preferential Flow Path Development and its Influence on Long-Term PRB Performance: Column Study. *Journal of Contaminant Hydrology*, 66, 161-178.

Kapoor, A. and Viraraghavan, T. (1997). Nitrate Removal from Drinking Water. *Journal of Environmental Engineering*, 123, 4, 371-380.

Keeny, D. R., and Follett, R. F., (1991). Managing Nitrogen for Groundwater Quality and Farm Profitability: Overview and Introduction. *Soil Science Society of America*, 357.

Kesseru, P., Kiss, I., Bihari, Z. and Polayak, B. (2003). Biological denitrification in a continuous-flow pilot bioreactor containing immobilized *Pseudomonas butanovora* cells. *Biosource Technol.*, 87, 75-80.

Kerri, L.C. and Flora, J.R.V. (1998). An Evaluation of Two Cathode Materials and the Impact of Copper On Bioelectrochemical Denitrification. *Water Research*, 32, 1, 63-70.

Koenig, A. and Liu, L. H. (2001). Kinetic Model of Autotrophic Denitrification in Sulphur Packed-Bed Reactors. *Water Research*, 35, 8, 1969-1978.

Koike, I. And Hattori, A. (1978). Denitrification and Ammonia Formation in Anaerobic Sediments. *Appl. Environ. Microbiol.*, 35, 278-282.

Krantzenstein, K. (1982). Apparatus and Methods for Denitrifying Water. *Patent Application 3121395, Dec. 16, 1982,* West Germany.

Kross, B.C., Hallberg, G.R., Bruner, D.R., Cherryholmes, K., and Johnson, J.K., (1993). The Nitrate Contamination of Private Well Water in Lowa. *Am. J. of Public Health,* 83, 270-272.

Kruithof, J. C., and Koppers, H.M.M., (1989). Experiences with Groundwater Treatment and Disposal of the Eliminated Substances in the Netherlands. *Aqua.* 38, 207-216.

Kurt, M., Dunn, I. J. and Bourne, J. R. (1987). Biological Denitrification of Drinking Water Using Autotrophic Organisms with H_2 in a Fluidized-bed Biofilm Reactor. *Biotechnol. Bioeng.*, 29, 493-501.

La Motta Diaz, E. and Andrade Salgado, A. (1985). Autotrophic Denitrification Using Sulfides. *Politecnica*, Quito, Ecuador, 10, 65-76.

Lavania, A. (2003). Zero-Valent Iron Assisted Autotrophic Denitrification: Optimal Reactive Media Composition and Buffering. M. Tech Thesis, Indian Institute of Technology, Kanpur, India.

LeCloirec, P. et al. (1985). Mathematical Model of Denitrification on Sulfur-Calcium Carbonate Filters. *Chem. Eng. Jour.*, Lausanne, Switzerland, 3, B9-B18.

Lee, D., Lee, I., Choi, Y. and Bae, J. (2001). Effects of External Carbon Source and Empty Bed Contact Time On Simultaneous Heterotrophic and Sulfur-utilizing Autotrophic Denitrification. *Process Biochemistry*, 36, 12, 1215-1224.

Lee, K.C. and Rittmann, B.E. (2003). Effects of pH and precipitation on autohydrogenotrophic denitrification using the hollow-fiber membrane-biofilm reactor. *Water Research*, 37, 1551-1556.

Lewandowski, Z.,R. Bakke, W.G. Characklis. (1987). Nitrification and Autotrophic Denitrification in Calcium Alginate Beads. *Wat. Sci. Tech.*, 19, 175-182.

Liu, L.H. and Koenig, A. (2002). Use of Limestone for pH Control in Autotrophic. Denitrification: Batch Experiments. *Process Biochemistry*, 37, 8, 885-893.

Mahony, F.R. and Associates Inc. (2000). http://www.frmahony.com

Manual of Water Supply and Treatment. Published by: Ministry of Urban Development, Government of India. 3rd Ed. 1999. 14-15.

Mansell, Bruce, O., Schroeder, Edward, D. (2002) Hydrogenotrophic Denitrification in Microporous Membrane Reactor. *Water Research,* 36, 19, 4683-4690.

Martin, G. (1982). Biological Denitrification of Water. *Patent Application* 79/10154, France.

Miller, D. W., Deluca, F. A. and Tessier, T. L. (1974). Ground-Water Contamination in the Northeast States. Washington, D. C.: Environmental Protection Agency, EPA-660/2-74-056.

Mirvish, S.S., Grandjean, A.C., Moller, H., Fike, S., Maynard, T., Jones, L., Rosinsky, S. and Nie, G. (1992). N-nitrosoproline Excretion by Rural Nebraskans Drinking Water of Varied Nitrate Content. *Cancer Epidemiol, Biomarkers & Prev.*, 1(6), 455-461. http://www.cheec.uiowa.edu/nitrate.

Mora, F.R., Giner, G.F., Andara, A.R., and Esteben, J.L. (2003). Effect of Organic Carbon Shock Loading on Endogenous Denitrification in Sequential Batch Reactors. *Biosource Technology,* 88, 215-219.

Moses, C.O., Nordstrom, D.K., Herman, J.S. and Mills, A.L. (1987). Aqueous Pyrite Oxidation by Dissolved Oxygen and by Ferric iron. *Geochim. Cosmochim. Acta,* 51, 1561-1571.

Murphy, A.P. (1991). Chemical Removal of Nitrate from Water. *Nature*, 350, 223-225.

Mueller, D.K., Hamilton, PA. , Helsel, D. R., Hitt, K. J., and Ruddy, B. C., (1995). Nutrients in Ground Water and Surface Water of the United States-An Analysis of the Data Through 1992. *U.S. Geol. Survey Water Res. Investigations Report.* 95-4031 .

Nakajima, M., Hayamizu, T. and Nishimura, H. (1984). Effect of Oxygen Concentration On the Rates of Denitrification and Denitrification in the Sediments of An Eutrophic Lake. *Water Research*, 18(3), 335-338.

Nilsson, I. and Ohlson, S. (1982). Columner Denitrification of Water by Immobilized *Pseudomonas denitrificans* cell. *European Jour. Appl. Microb. Biotech.*, 14, 86-90.

Oh, S E., Yoo, Y.B., Young, J.C. and Kim, I. S. (2001). Effect of Organics On Sulfur-utilizing Autotrophic Denitrification Under Mixotrophic Conditions. *Jour. of Biotechnol.*, 92(1), 1-8.

Overath, H., Hussmann, A. and Haberer, K. (1986). Biological Nitrate Removal by *Thiobaciccus Denitrificans* Using Elemental Sulfur Fixed on Activated Carbon Electron Donor. *Vom Wasser*, Weinheim, West Germany, 66, 59-83.

Palomares, A.E., Prato, J.G., Marquez, F., and Corma, A. (2003). Denitrification of Natural Water on Supported Pd/Cu Catalysts. *Appl. Catal. B: Environmental,* 41, 3-13.

Paul, E.A., Clark, F.E. (1996). Soil Microbiology and Biochemistry. *Academic Press, San Diego,* 2nd Edition

Pauwels, H., Kloppmann, W., Foucher, J.C., Martelat, A., and Fritsche, V. (1998). Field tracer test for Denitrification in a Pyrite-Bearing Schist Aquifer. *Appl. Geochem.*, 13, 6, 767-778.

Pauwels, H., Foucher J.C., Kloppmann, W. (2000). Denitrification and Mixing in a Schist Aquifer: Influence on Water Chemistry and Isotopes. *Chemi. Geol.*, 168, 307-324.

Payne, W. J., (!973) Reduction of Nitrogenous Oxides by Microorganisms. *Bacteriol. Review.*37, 409-452.

Peel, J. W., Reddy, K.J., Sullivan, B. P., and Bowen, J. M. (2003). Electrocatalytic Reduction of Nitrate in Water. *Water Research,*37, 10, 2512-2519.

Philipot, J. M., Chaffange, F. and Pascal, O. (1985). Denitrification Biologique: le Point sur un an de Fonctionnement de la Station d'eragny. *Water Supply*, 3, 93-98.

Pintar, A. (2003). Catalytic processes for the Purification of Drinking Water and Industrial Effluents. *Catalysis Today*, 77, 451-465.

Ploz, B. G., Jobbagy, A. and Grady, C. P. L. (2003). Factors Influencing Deterioration of Denitrification by Oxygen Entering an Anoxic Reactor through the Surface. *Water Research*, 37, 853-863.

Postma, D. and Boesen, C. (1991). Nitrate Reduction in an Unconfined Sandy Aquifer: Water Chemistry, Reduction Processes, and Geochemical Modeling. *Water Resource Research*, 27, 8, 2027-2045.

Qu, J., Fan, B., Liu, S., Lei, P. and Liu, H. (2001). Autotrophic Denitrification of Groundwater by Electrochemical Process. *Huan Jing Ke Xue*, 22, 6, 49-52. http://www.ncbi.nlm.nih.gov/entrez

Qu, J., Fan, B., Ge, J. and Liu, H. (2002). Denitrification of Drinking Water by a Combined Process of Heterotrophication and Electrochemical Autotrophication. *J. Environ. Sci. Health -Part A Toxic/Hazardous Substances and Environmental Engineering*, 37, 4, 651-665.

Rauschmaier, R. and Barotke, D. (1985). Birchwood as a Hydrogen Donor in The Microbial Denitrification of Water. *Material und Organism*, Berlin, West Germany, 20, 253-264.

Reddy, K. J., Lin, J., (2000). Nitrate Removal from Groundwater using Catalytic Reduction. *Water Resources,* 34, 3, 995-1001

Richard, Y., et al. (1980). Denitrification of Water for Human Consumption. *Progress in Water Technology*, Oxford England, 12, 173-191.

Roennefahrt, K. (1985). Biological Denitrification in Fixed-bed Reactors. *Vom Wasser*, 65, 271-285.

Rogalla, F., Lurminat, G. D. E., Coutelle, J., and Godart, H., (1990). Experience with Nitrate Removal Methods from Drinking Water. In *Proceedings of NATO Advanced Research Workshop, University of Nebraska, Lincoln, Nebraska.*

Saffigana, P. G., Keeney, D. R., (1977). Nitrate and Chloride in Groundwater under Irrigated Agriculture in Central Wisconsin. *Ground Water,* 15, 170-177

Siemens, J., Hass, M., and Kaupenjohann, M. (2003). Dissolved Organic Matter induced Denitrification in subsoils and aquifers. *Geoderma*, 113, 253-271.

Sliekers, A.O., Derwort, N., Campos Gomez, J. L., Strous, M., Kuenen, J.G. and Jetten, M.S.M. (2002). Completely Autotrophic Nitrogen Removal Over Nitrite in One Single Reactor. *Water Research*, 36, 10, 2475-2482.

Smith, R.L., Ceazan, M.L. and Brooks, M.H. (1994). Autotrophic, Hydrogen-Oxidizing, Denitrifying Bacteria in Groundwater, Potential Agents for Bioremediation of Nitrate Contamination. *Appl. Environ. Microbiol.*, 60, 6, 1949-1955.

Smith, R.L. and Duff, J.H. (1988). Denitrification in a Sand and Gravel Aquifer. *Appl. Environ. Microbiol.*, 54, 5, 1071-1078.

Sontheimer, H., et al. (1987). Biological Denitrification of Water with Minimum Post treatment. *Patent Application 3603123*. West Germany.

Soares, M. I. M. (2002). Denitrification of Groundwater with Elemental Sulfur. *Water Research*, 36, 5, 1392-1395.

Spalding, R. F., Gormly, J. R., Curtiss, B. H., and Exner, M. E. (1978). Nonpoint Nitrate Contamination of Ground Water in Merrick County. Research Supported by the Central Platte Natural Resources District and the Old West Commission Project No. 10670217.

Schipper, L.A., Barkle, G.F., Hadfield, J.C., Vojvodic-Vukovic, M., and Burgess, C.P. (2004). Hydraulic Constraints on the Performance of a Groundwater Denitrification Wall for Nitrate Removal from Shallow Groundwater. *Journal of Contaminant Hydrology*, 69, 263-279.

Trudell, M.R., Gillham, R.W. and Cherry, J.A. (1986). An In-situ Study of the Occurrence and Rate of Denitrification in a Shallow Unconfined Aquifer. *Jour. Hydrol.*, 83, 251-268.

Till, B.A., Weathers, L.J. and Alvarez, P.J. (1998). Fe (0)-Supported Autotrophic Denitrification. *Environ. Sci. Technol.*, 32, 634-639.

U. S. Geological Survey. (1990). Digital Data Used to Relate Nutrient Inputs to Water Quality in the Chesapeake Bay Watershed, Version 2.0-Septic Tanks, *USGS Open-File Report 01-251*. http://md.water.usgs.gov/gis/chesbay/sparrow2/doc/septic90.htm.

Van der Hoek, J. P. and Klapwijk, A. (1988). The Use of a Nitrate Selective Resin in the Combined Ion Exchange/ Biological Denitrification Process for Nitrate Removal from Groundwater. *Water Supply*, 6, 57-62.

Vidal, S., Rocha, C. and Galvao, H. (2002). A Comparison of Organic and Inorganic Carbon Controls Over Biological Denitrification in Aquaria. *Chemosphere*, 48, 445-451.

Vitousek, P. M., Aber, J. D., Howarth, R. W., Likens B. E. Matson, P. A. Schindler, D. W., Schlesinger, W. H., and Tilman, D.B., 1997. Human alterations of the Global Nitrogen Cycle: Sources and Consequence. *Ecol. Appl.* 7, 3, 737-750

Vogel, J. C., Talma, A. S. and Heaton, T. H. E. (1980). Gaseous Nitrogen As Evidence for Denitrification in Groundwater. *Jour. Hydrol.*, 50, 191-200.

Walton, B. (1951). Survey of Literature Relating Infant Methemoglobinemia Due to Nitrate Contaminated Water. *Am J Public Health*. 41, 986–996.

Ward, M.H., Zahm, S.H., Blair, A. (1994). Dietary factors and Non-Hodgkin's Lymphoma. *Cancer Causes Control.* 5, 5, 422 – 32.

Watson, K.S., Farell, F.P. and Anderson, J.S. (1967). The Contribution from the Individual Home to the Sewer System. *J. of Water Pollution Control Federation*, 39, 2039.

Welander, U., and Mattiasson, B. (2003). Denitrification at low temperatures using a suspended carrier biofilm process. *Water Research*, 37, 10, 2394-2398.

Young, C.P. and Gray, E.M. (1978). Nitrate in Ground Water. *Water Res. Cent.*, Medmenham, Tech. Rep. 69.

Yull-Rhee, G. and Futts, G. W. (1978). Wastewater Denitrification with One Carbon Compounds As Energy Source. *J. Water Pollution Control Federation,* 50, 2111-2119.

Zhang, T.C. and Lampe, D.G. (1998). Sulfur: Limestone Autotrophic Denitrification Processes for Treatment of Nitrate-Contaminated Water: Batch Experiments. *Water Research*, 33, 3, 599-608.